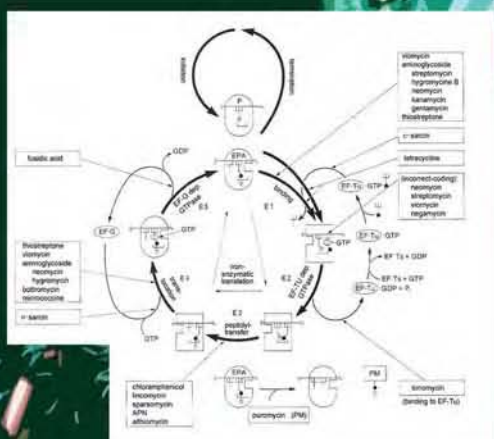


# Plant Toxicology

*Fourth Edition*



edited by  
**Bertold Hock**  
**Erich F. Elstner**

# **Plant Toxicology**

*Fourth Edition*

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# Preface

Plant toxicology is dealing with poisons causing harmful effects in plants. Not only humans and animals, but also plants are affected by a multitude of toxins. Therefore plant toxicology is concerned with damages, which are caused by toxic agents, either accidentally or deliberately. Considering the growing number of environmental compounds interfering with plant metabolism and development, and keeping in mind the role of plants as primary producers of food, it is surprising that the term toxicology has been confined almost exclusively to humans and animals. If recent damages such as forest diebacks are taken into account, it is clear that plant toxicology represents an important branch in biological sciences.

Understanding of toxic processes in plants requires a detailed knowledge of molecular events when toxic compounds as well as elicitors during host–pathogen interactions bind to their molecular targets. However, toxicology involves the entire series of phases that are relevant for the toxic process, i.e. exposure to toxic material, uptake, distribution, metabolism and finally secretion. These topics are central to this book.

Another focus is the recognition and possible prevention of damage, caused by environmental pollutants. Quantification of damage is therefore crucial. But plant toxicology also deals with negative effects, which are intended. Agriculture and horticulture provide many examples, such as the use of herbicides. Questions concerning the uptake, metabolism and detoxification have to be solved before suitable and justifiable applications can be considered.

Although exogenous compounds, which normally do not occur in the metabolism of plants (xenobiotics) are central to this book, it should be

noted that also endogenous compounds of the organism can become harmful when certain thresholds are exceeded. Such effects may result from over-fertilization and are therefore taken into account. Even physical factors such as ionizing radiation or detrimental effects of biogenic origin such as infestation with parasites have to be considered if damage arises similar to the impact of xenobiotics.

The boundaries of plant toxicology are relatively wide. They are primarily determined by practical aspects. This book should also help to classify observed damage and, if possible, identify. The limitation on eukaryotic plants as potential target groups will meet practical interests.

Methods of plant toxicology originate primarily from chemistry and biochemistry. Chemical analysis provides mainly the methods, biochemical techniques contribute to the elucidation of action mechanisms and metabolism of toxic compounds. Progress in toxicology largely depends on the development of new methods and techniques.

It is not sufficient to see plants as isolated organisms. The consideration of the ecological context is an important requisite for the evaluation and abolishment of toxic influences. Basics of biological knowledge are essential and provided where needed.

The editors would like to thank Marcel Dekker, Inc., particularly Theresa Stockton, who edited and guided the book throughout production. Indeed we wish to thank the entire staff for their understanding, encouragement, and practical help.

*Bertold Hock  
Erich F. Elstner*

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# 1

## Characteristics of Plant Life: Hazards from Pollutants

**Bertold Hock and Nicola M. Wolf**

*Technische Universität München, Freising, Germany*

### **I. GREEN PLANTS AS PHOTOAUTOTROPHIC ORGANISMS**

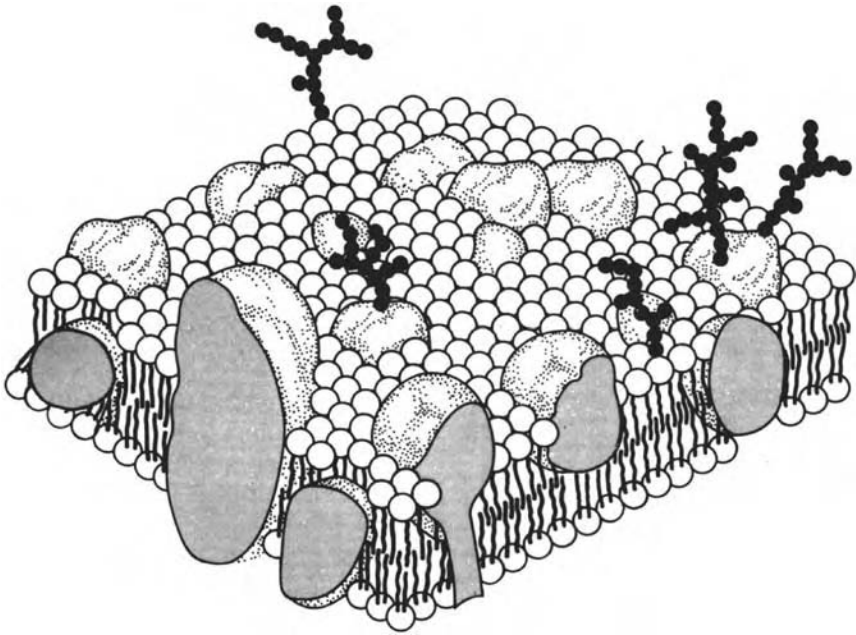
Living organisms are characterized by their extraordinary complexity. It is manifested in the higher plant by a hierarchy of structures comprising organs, tissues, and cells. The following chapters provide an introduction to plant organization starting with the cell as the smallest elementary unit. Emphasis is laid upon plant-specific features, which are discussed with respect to their susceptibility to environmental contaminants.

### **II. FUNCTIONAL ORGANIZATION OF THE CELL**

#### **A. Role of Membranes**

Plant cells as eukaryotes are characterized by their compartmentation into membrane-enclosed reaction spaces. This structure separates different metabolic pathways but at the same time allows a grouping of connected biochemical functions. This process allows sophisticated regulations.

The borders of compartments are composed of biomembranes (Fig. 1). These thin and highly flexible structures determine the architecture of biological systems. Biomembranes are flat, asymmetrical structures, which are closed and usually topologically equivalent to the surface of a sphere or a torus. Along with the basic composition of lipids and proteins there are variety of individual compositions. The ratio of proteins to lipids varies within a range of 1:4 to 4:1. Both components are held together by

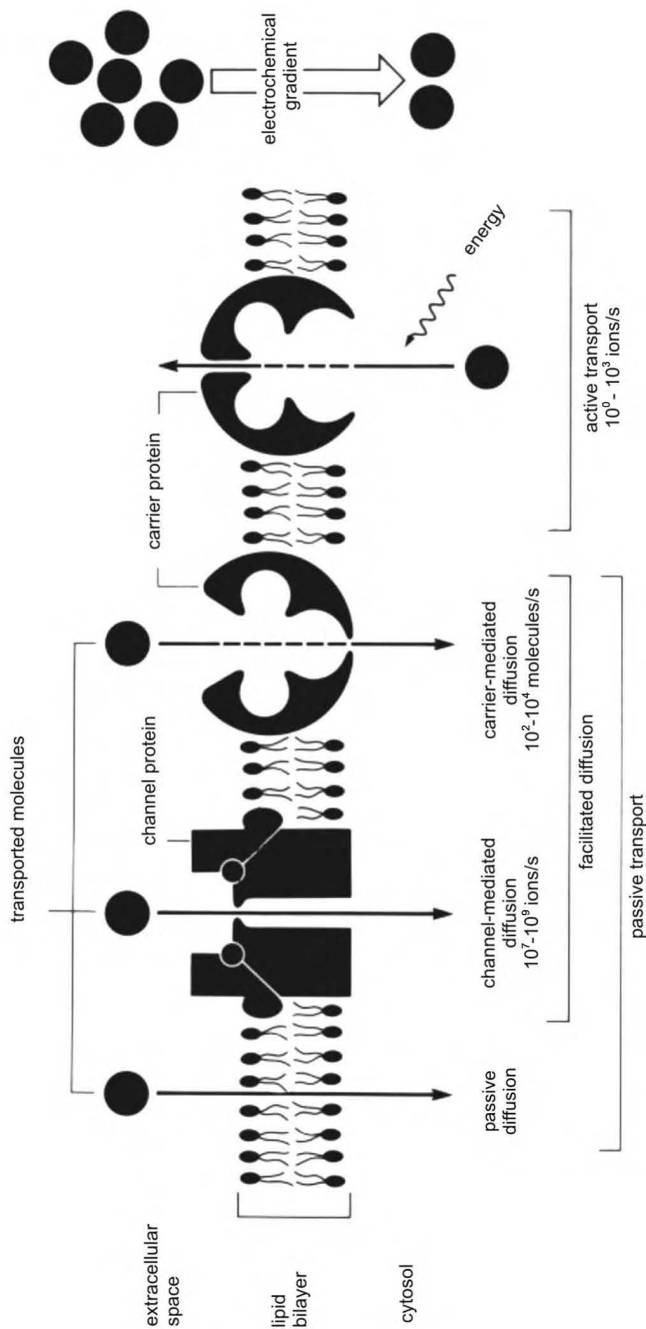


**Figure 1** Biomembrane with integral and peripheral membrane proteins (1). The protruding oligosaccharide chains belong to glycoproteins and glycolipids.

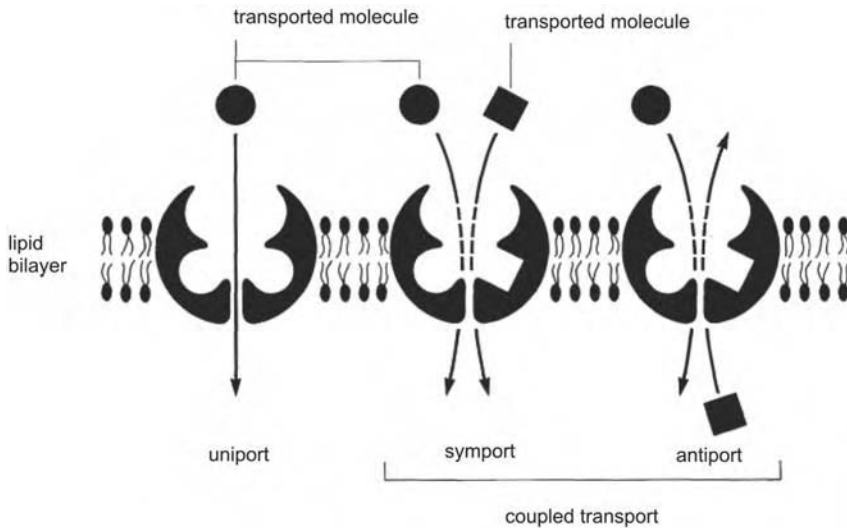
hydrophobic interactions. The lipids form a bimolecular layer, which serves as a barrier and prevents the passage of polar molecules. Integral proteins penetrate completely or at least partly the lipid bilayer. Conspicuous examples are the chlorophyll a/b-binding protein of chloroplast membranes and the adenosine triphosphate (ATP) synthase of mitochondrial and chloroplast membranes. Peripheral proteins bind at the membrane surface to integral proteins from which they can be detached easily by appropriate solvents. They include clathrin, spectrin, and ankyrin of the plasma membrane. Usually membrane proteins have mediating functions: they serve as receptors, ion channels, ATP-powered pumps, or transporters. In many cases enzymatic activities are crucial for the function. This explains why the two sides of the membrane bilayer are usually different.

Transport through a biomembrane uses one of the three following mechanisms (Fig. 2):

1. Passive diffusion: Only a few substances are able to penetrate the lipid bilayer. Examples are gases such as oxygen ( $O_2$ ), nitrogen ( $N_2$ ), or carbon dioxide ( $CO_2$ ) and some small, uncharged molecules such as ethanol,



**Figure 2** Scheme of the membrane transport (2). Passive transport follows an electrochemical gradient as free diffusion or facilitated diffusion and is mediated by transport proteins. In contrast, active transport requires adenosine triphosphate- (ATP)-powered pumps and moves ions or small molecules uphill against the electrochemical gradient.



**Figure 3** Scheme of transport proteins, which act as uniporters, symporters, or antiporters (2).

urea, or benzene, which dissolve easily in the lipid bilayer. In principle this also holds true for water ( $\text{H}_2\text{O}$ ) although its diffusion may be accelerated by transport proteins (aquaporins). Passive diffusion of  $\text{H}_2\text{O}$  through the biomembrane is crucial for osmosis. In contrast, lipid bilayers are practically impermeable to charged molecules.

2. **Facilitated diffusion (catalyzed diffusion)**: Similar to passive diffusion this mechanism does not require energy and leads only to a concentration equilibrium. However, transport proteins (channels and transporters) are required for this type of membrane transport. Three options are available (Fig. 3): (a) unidirectional transport (uniport), which moves only one kind of molecule; (b) symport (cotransport), in which two molecules or ions are transported in the same direction, and one of them, for instance,  $\text{H}^+$ , follows a concentration gradient; (c) antiport, in which two molecules or ions move in opposite directions, one of them following a concentration gradient. Both antiporters and symporters mediate coupled reactions in which the energetically unfavorable reaction is coupled to an energetically favorable reaction.

3. **Active transport**: The process pumps ions or small molecules through a membrane against a chemical concentration gradient and/or electric potential. This ATP-powered transport moves ions such as  $\text{H}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Na}^+$  in one direction. It is mediated by adenosine triphosphatases

(ATPases). The required energy is released by the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate ( $P_i$ ). This active transport indirectly drives symport and antiport.

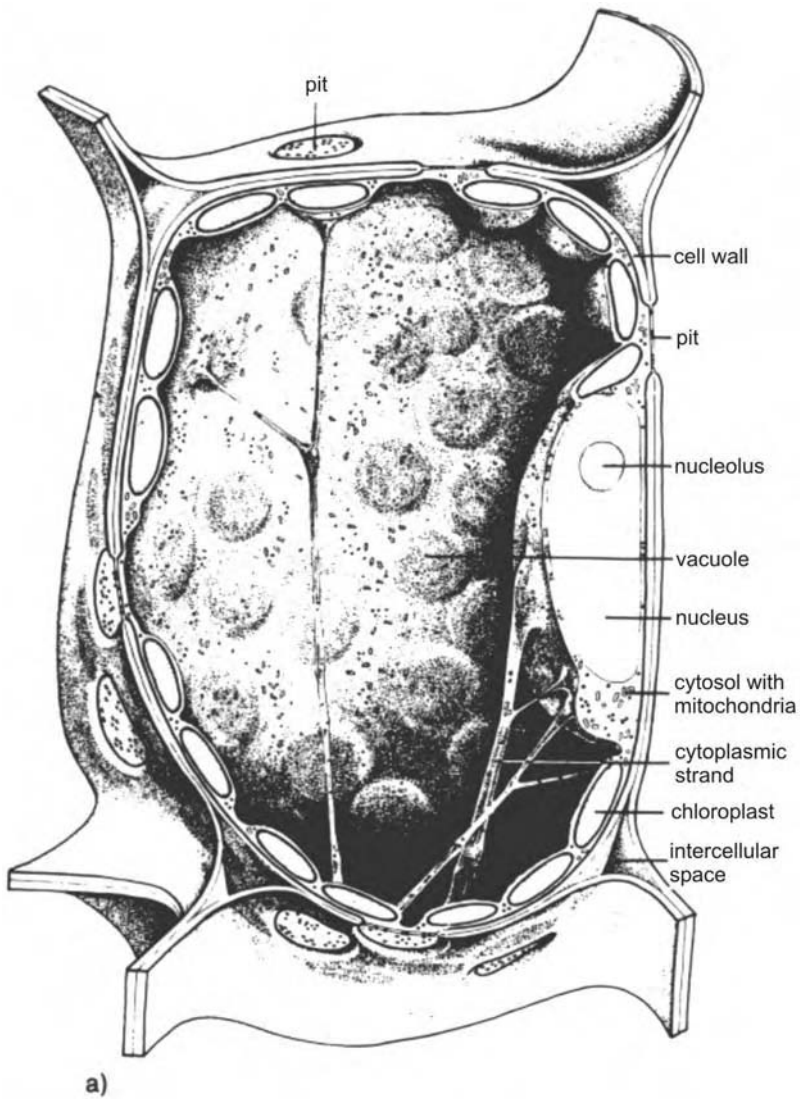
The compartmentation of cells creates huge membrane areas, which offer extended targets to environmental compounds and toxins approaching and penetrating the cells. Binding of a variety of substances can lead not only to impairment of individual functions, but also to destruction of whole compartments. The abolition of compartmentation always leads to cell death.

Compartmentation entails a separation of protoplasmic and nonprotoplasmic spaces: the plasmalemma separates the protoplast from the cell wall at the outside, the tonoplast from the vacuole at the inside (Fig. 4). The plasmalemma (plasma membrane) allows the movement of solutes into the protoplast as well as outward. In addition, it is involved in signal transduction between the outside and inside and mediates hormonal as well as light effects. This allows the cell to detect changes of its environment and react accordingly. For this purpose several receptor types as well as redox chains are available. A prominent component of the plasmalemma is the  $H^+$ -ATPase. This transport ATPase plays a crucial role in the uptake of nutrients and pH regulation. By means of this ATP-driven  $H^+$  pumping the membrane becomes energized, and the electrochemical potential for the import of solutes through ion channels and carriers is maintained by ATP hydrolysis. This function is taken in animal cells by the sodium pump ( $Na^+/K^+$ -ATPase). Whereas in this case the pump is usually coupled to  $K^+$  import and  $Na^+$  export, proton coupling is used by plants. The proton pump transports a single  $H^+$  for each hydrolyzed ATP and creates in this way a large electrical potential up to  $-300$  mV (inside the membrane negative) as well as a proton gradient. The values reach a pH of c. 7.1 at the inner side of the membrane and at the outside values between 4.5 and 5. A comparison of the different strategies is illustrated by Fig. 5.

Cells with intensive active transport such as root hairs require between 25% and 50% of their total cellular ATP to keep their proton pumps running. In addition hydrogen adenosine triphosphatases ( $H^+$ -ATPases) play an important role in cell elongation (cf. acid growth theory: Chapter 1, 2D structure and function of the cell wall).

The plasma membrane contains a multitude of further membrane proteins, depending on the specific cell. In addition to  $H^+$ -ATPases, cation and anion channels as well as carriers for sucrose, nitrate, and amino and a multitude of hormone and blue light receptors have been identified. Even many pollutants act on components of the plasmalemma, especially on  $H^+$ -ATPases. Many xenobiotics also enter the cell, where they are taken up directly or after conjugation to glutathione into the vacuole. In other cases





**Figure 4** Structure of a plant cell: (a) Scheme (3); (b) electron micrograph of a tobacco mesophyll cell. ER, endoplasmic reticulum. (With kind permission of Prof. Katherine Esau, University of California.)

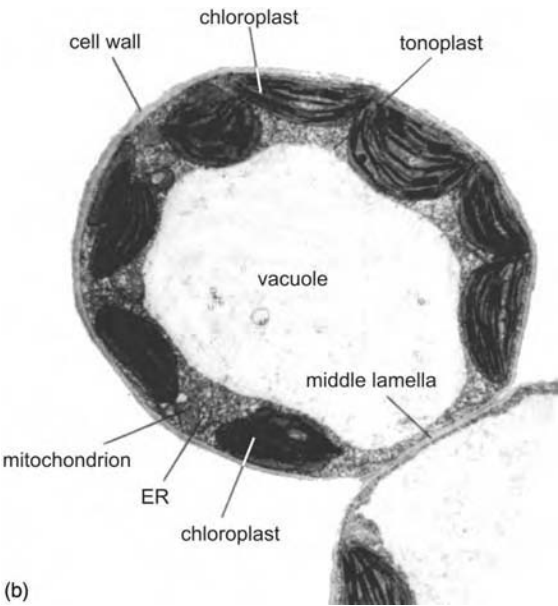


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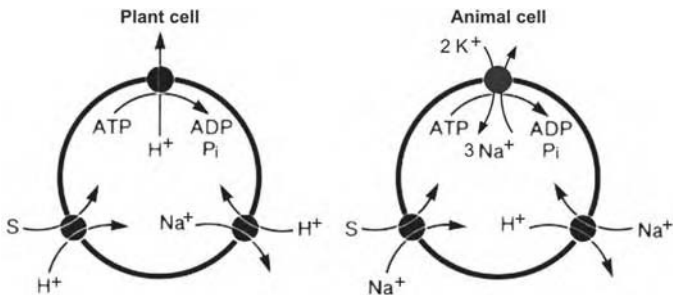


Figure 5 Different strategies of plant and animal cells regarding the uptake of solute (S) and pH regulation. ATP, adenosine triphosphate; ADP, adenosine diphosphate;  $P_i$ , inorganic phosphate. (From Ref. 4.)

cytochrome P450-dependent hydroxylations occur, followed by further metabolic steps.

According to the principle of hierarchic structuring the protoplast is divided into several compartments. The membrane-enclosed spaces are organelles in a narrower sense. There are organelles bounded by two

membranes (cell nucleus, mitochondria, and plastids) and organelles surrounded by a single-membrane envelope (such as endoplasmic reticulum, dicytosomes, lysosomes, peroxisomes, glyoxysomes, and microbodies). These organelles are embedded into the cytosol, which also contains additional particles (organelles in a wider sense such as ribosomes as well as fibrous elements belonging to the cytoskeleton). Sometimes the traditional grouping of the protoplast into cell nucleus and cytoplasm is used.

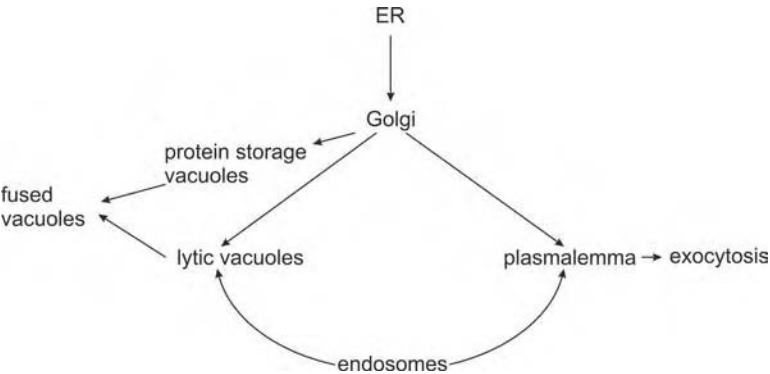
With respect to cell compartmentation the following rules are important: an envelope composed of two membranes separates plasmatic from other plasmatic spaces (P spaces). The P spaces are characterized by their potential to synthesize nucleic acids and/or proteins. The delimitation of the nucleus, mitochondria, and plastids against the cytosol falls into this group. A delimitation by a single membrane separates plasmatic from endoplasmatic spaces (E spaces). The latter spaces are not capable of nucleic acid or protein synthesis. The cell wall and vacuole are nonplasmatic spaces, as are the lumen of the endoplasmic reticulum (ER), dicytosomes, lysosomes, and microbodies. The symbiont theory of organelle evolution explains these distinctions: mitochondria and plastids are derived from prokaryotic cells that have colonized as endosymbionts, the progenitors of modern eukaryotes.

Cell membranes are subjected to remarkable dynamics, to which membrane flow substantially contributes. Membrane flow is mediated by an intensive vesicle stream within the cytosol. There are several options, depending on the respective cell: the pathway from the ER, which is connected to the nuclear envelope, via the Golgi apparatus to the plasmalemma (exocytosis) or to the lysosomal compartment. On the other hand, components of the plasmalemma, e.g., receptors, are recycled by endocytosis. By means of membrane flow large amounts of newly synthesized fatty acids and lipids are transported from the ER to the plasmalemma. Figure 6 gives an overview of the path of vesicle streams.

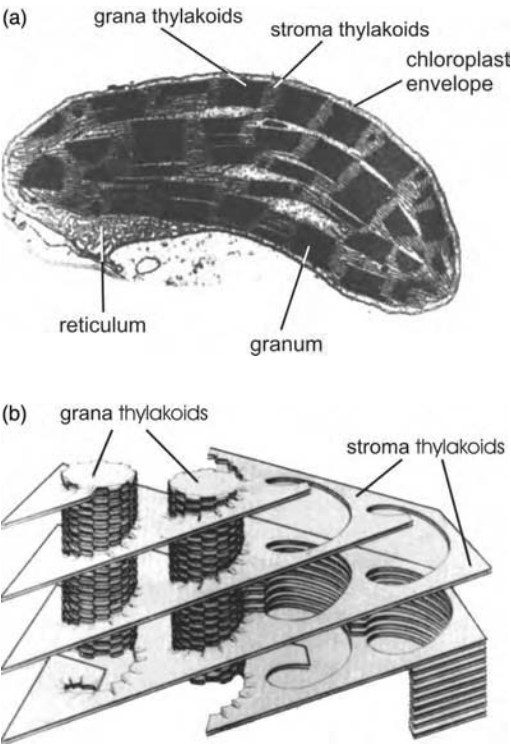
The plant cell differs from the animal cell in the existence of three additional compartments, plastids, vacuole, and cell wall, whereas the other structures are generally very similar. These additional components provide specific targets for pollutants, but on the other hand remarkable indifference to certain xenobiotics that have dramatic effects on animals and humans prevails.

## **B. Chloroplasts, the Photosynthetic Organelles**

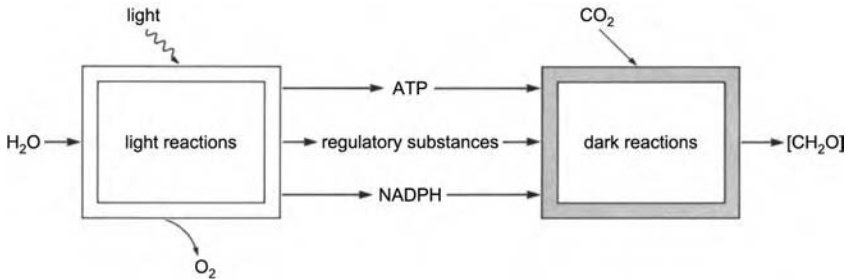
Green plants as photoautotrophic organisms utilize the energy of sunlight for the synthesis of high-energy compounds. This transformation of energy takes place in the chloroplasts (Fig. 7a). Here the light energy is used for



**Figure 6** Membrane flow between different compartments. ER, endoplasmic reticulum.



**Figure 7** Structure of chloroplasts: (a) Electron micrograph of a mesophyll cell from a corn leaf (5). (b) Arrangement of thylakoid membranes (scheme) (6).



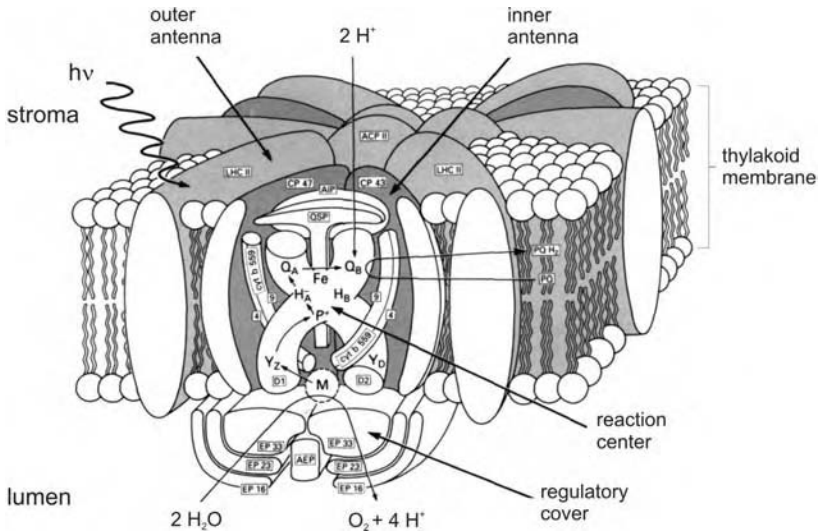
**Figure 8** Contribution of light and dark reactions of photosynthesis to  $\text{CO}_2$  assimilation (7). ATP, adenosine triphosphate; NADPH, reduced nicotinamide adenine dinucleotide.

the generation of the assimilatory power for the dark reactions, a historic term for the synthesis of carbohydrates from  $\text{CO}_2$ , for which light energy itself is not required. Actually the light-driven reactions provide specific regulatory substances, which restrict the operation of the dark reactions to the light period. Regulation involves the ferredoxin/thioredoxin system, Fig. 8 shows this connection. A light-driven reaction chain (light reactions I and II) requiring intact thylakoids (an endomembrane system of chloroplasts; Fig. 7b) generates the assimilatory power provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ATP.

### 1. Reduced Nicotinamide Adenine Dinucleotide Phosphate

The electrons required for the reduction of the coenzyme, are provided by an electron transport chain. It accepts electrons from a water-splitting complex ( $\text{H}_2\text{O} \rightarrow \frac{1}{2} \text{O}_2 + 2\text{H}^+ + 2\text{e}^-$ ) and moves them with the aid of light-activated “pump station,” photosystem II (PS II) and I (PS I), to nicotinamide adenine dinucleotide phosphate (NADP). The photoreceptor of the two photosystems is the green pigment chlorophyll a, surrounded by several so-called antenna pigments. A destruction of these pigments, for instance, during bleaching reactions, interferes with photosynthesis.

The photosynthetic pigments are embedded in a multiprotein complex. Photosystem II, which catalyzes the oxidation of  $\text{H}_2\text{O}$  and the reduction of the electron acceptor plastoquinone, contains the homologous polypeptides  $\text{D}_1$  and  $\text{D}_2$ . Figure 9 shows a model of photosystem II. The  $\text{D}_1$  polypeptide has a fast turnover. Under high light intensities, which exceed the adaptive level, the degradation is faster than the speed of repair, and photoinhibitor



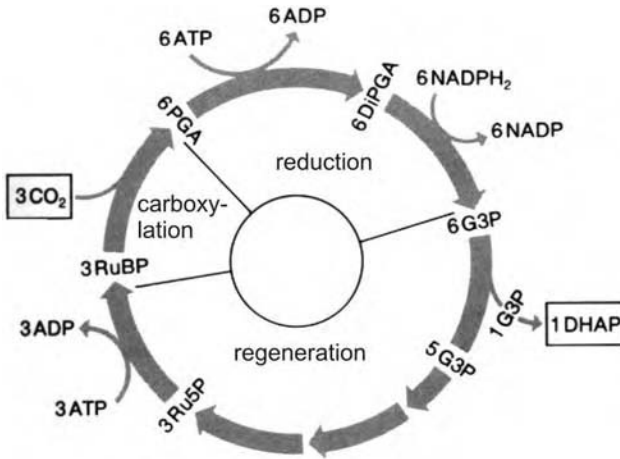
**Figure 9** Functional organization of photosystem II (8). P, chlorophyll P680, the primary electron donor;  $H_A$ , pheophytin, the primary electron acceptor;  $H_B$ , a second pheophytin;  $Q_A$  and  $Q_B$ , the first and second quinone electron acceptor; Fe, a nonheme iron protein;  $Y_Z$ , a tyrosine residue as first electron donor for P;  $Y_D$ , a second tyrosine residue; M, a manganese-containing component, which is required for the  $O_2$  formation; cyt b559, cytochrome b559 heterodimer;  $D_1$  and  $D_2$ , subunits of the reaction center; QSP, quinone shielding protein; AIP, accessory intrinsic protein; EP 33, 23, and 16, extrinsic proteins of the regulatory shielding; AEP, accessory extrinsic protein; CP 47 and 43, chlorophyll-binding proteins of the proximal antenna; ACP II, accessory chlorophyll-binding proteins of the distal antenna; LHC II, the chlorophyll a/b light harvesting complex.

takes place. The light damage of PS II results in inhibition of  $O_2$  production, electron transfer reactions, and finally  $CO_2$  fixation.

## 2. Adenosine Triphosphate

During the electron flow from water to oxidized NADP ( $NADP^+$ ) protons are expelled into the lumen of the thylakoids and an electrochemical potential is generated between the inside and the outside of these membranes. This energy difference is used for ATP synthesis (photophosphorylation), in which protons flow through the channels of the ATP synthase (coupling factors) and leave the lumen.

The NADPH and ATP are used for several syntheses, which take place in a second, light-independent reaction sequence (dark reactions) in the stroma of chloroplasts. Most important is the reduction of



**Figure 10** The reductive pentose phosphate cycle (Calvin cycle) (7). RuBP, ribulose-1,5-bisphosphate; PGA, 3-phosphoglycerate; DiPGA, 1,3-diphosphoglycerate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; Ru5P, ribulose-5-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH<sub>2</sub>, reduced nicotinamide adenine dinucleotide phosphate.

phosphoglycerate to glyceraldehyde. In addition, nitrate assimilation (nitrite reductase and glutamate synthase cycle) as well as sulfate assimilation of the green plant are coupled to photosynthesis.

Figure 10 gives an overview of the primary mechanism of CO<sub>2</sub> assimilation. It is integrated into the reductive pentose phosphate cycle (Calvin cycle) involving the carboxylation of ribulose-1,5-bisphosphate (RuBP) by the enzyme ribulose bisphosphate carboxylase (RUBISCO), releasing two molecules of 3-phosphoglycerate (PGA) as the key reaction. This reaction needs neither ATP nor NADPH. The next steps enclose the reductive phase of the cycle. Here ATP and NADPH are used for the reduction of 3-phosphoglycerate to glyceraldehyde-3-phosphate (G3P). This compound is partly used as a carbon source for the synthesis of starch in the chloroplast or is exported to the cytosol for subsequent sucrose synthesis. Part of it is consumed for the regeneration of the starter molecule ribulose-1,5-bisphosphate in a regenerative phase, involving a series of isomerization, condensation, and rearrangement reactions requiring ATP. Thus green plants, in contrast to heterotrophic organisms, are independent of the supply of energy-rich molecules from the outside. This system has far-reaching consequences for the organization of the green plant on all structural and functional levels. The photosynthetic apparatus is not only



a major target of many pollutants is also increasingly a target for several newly developed herbicides. It is clear that a responsible application of these compounds depends on a highly specific action, which is restricted to the weeds to be killed and does not increase general environmental pollution. A primary requirement to reach this goal is detailed knowledge of the biochemical basis of photosynthesis.

### C. Composition and Function of the Vacuole

The vacuole (Fig. 4b) accounts for up to 90% of the cell volume in an adult plant cell. It arises during cellular growth from the fusion and increase of several small vacuoles, which belong to the lysosomal compartment. The vacuole is filled with the cell sap, an acidic solution composed of inorganic and organic ions, sugars, amino acids, and repellent proteins, as well as a variety of secondary plant metabolites (for instance, phenols, isoprenoids, alkaloids, glycosides) in changing composition. The contact of the protoplast with the aggressive cell sap is prevented by the tonoplast.

The vacuole takes a unique position among the plant cell compartments with regard to its multifunctional role. In addition to storing water and temporarily storing reusable substances, it serves as a depot for metabolic end products as well as for xenobiotics, e.g., in the form of glutathione-S conjugates. Several of these compounds serve as allelochemicals for protection and defense.

Some of the solutes are second messengers. For instance,  $\text{Ca}^{2+}$  ions have regulatory functions after their release into the cytosol. Similar to the plasmalemma the tonoplast contains a multitude of transport ATPases, ion channels, and carriers, which regulate the uptake into the vacuole and the release into the cytosol. The  $\text{H}^{+}$ -ATPases and  $\text{H}^{+}$ -pyrophosphatases play an essential role. They form the  $\text{H}^{+}$  gradient between vacuole and cytosol, the driving force for the uptake of many compounds into the vacuole. The considerable pH differences between cytosol (7.1–7.5) and vacuole (4.5–6.0) or the outer medium (5–8) require a precise regulation of the cytosolic pH because most of the cytoplasmic processes are pH-sensitive. In addition to the considerable pH gradients the electric field across the plasmalemma leads to large proton fluxes. Mainly  $\text{H}^{+}$ -ATPases of the plasmalemma and tonoplast contribute to the constancy, because they remove protons from the cytosol under ATP consumption. On the other hand, the pH increase in the outer medium leads to a passive influx of  $\text{OH}^{-}$  ions and to a simultaneous synthesis of considerable amounts of organic acids such as malate and citrate to maintain the cytosolic homeostasis of the pH.

In addition to transport proteins there are trap mechanisms, which allow the uptake and storage of several secondary plant metabolites,



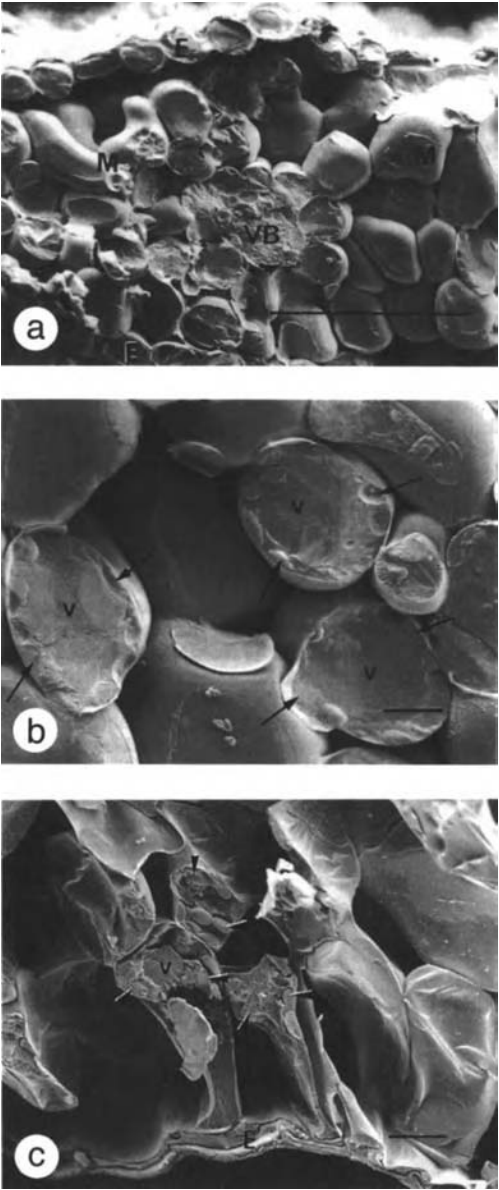
circumventing active uptake mechanisms. In the case of passive and facilitated diffusion fluxes into the vacuole are possible if the transported compounds are either transformed by further metabolic activities or bound to other cell sap components, e.g., phenols or ions, and therefore withdrawn from the diffusion equilibrium or transformed into nonconvertible forms. This includes isomer traps, which have been described for the uptake and storage of *o*-cumaric acid glucosides and acetylated glucosides (9).

Because of the large amounts of solutes the water potential of the cell sap is decreased toward the cytosol and the outer solution in the cell wall: the vacuole draws water from the outside through the semipermeable boundary membranes of the protoplast. The osmotically driven volume increase of the cell sap pushes the protoplast against the cell wall. This hydrostatic pressure is referred to as *turgor pressure*. The counterpressure of the elastically stretched cell wall (wall pressure) contributes to the unusual strength of plant cells, which in the absence of a skeleton system leads to the mechanical strength of unlignified tissues. The minimal investments by the plant are remarkable. In addition, the turgor provides the force necessary for the stretching the walls of a young cell.

Stress conditions such as drought, low temperature, and high salt concentration interfere with the intracellular water balance and therefore limit plant growth and yield. The accumulation of osmotically active, low-molecular-weight compounds such as sugar alcohols, proline-betaine, and glycine-betaine as osmolytes significantly contributes to the protection of plant cells.

Withdrawal of water caused by frost is particularly critical for turgor. Below defined temperatures, which are different for individual plants, extracellular formation of ice crystals is observed, especially in the subepidermal intercellular spaces, resulting in an initially reversible withdrawal of water from the cells. If sufficiently low temperatures are reached, a cell collapse takes place because of the strong dehydration (cf. Fig. 11). A collapse of the turgor also results from direct attacks on cell membranes. This effect can be used for the control of several pathogenic fungi by antibiotics.

A large central vacuole guarantees maximal contact of the protoplast to its surroundings. This is particularly important for photosynthesis: a minimal distance of chloroplasts to the gas phase reduces not only light absorption, but also diffusion resistance of the liquid phase to CO<sub>2</sub>. Finally the plant vacuole allows a cell volume that exceeds the average order of magnitude of an animal cell by one to two orders, keeping the energy and the investment to a minimum. The construction of the sessile plants is optimized for maximal contact to their surroundings, which is reached most advantageously by dendritic structures. An optimal partitioning and



**Figure 11** Influence of frost on a wheat leaf (10). A, B: controls at 14°C. C: frost at -9°C. The ice was removed by sublimation. Scanning electron micrograph of field samples: E, epidermal cells; v, vacuole; M, mesophyll cells; VB, vascular bundle. Measuring bars: A and C, 100 µm; B, 10 µm.

adaptation of the protoplast to this structure allow the plant to fill up to 90% of its volume with water within the vacuoles without impairing the contact areas of the protoplasm with its surroundings. It is no coincidence that mobile plant cells such as flagellated algae cells, but also sperm of higher plants (e.g., ferns), usually do not have vacuoles. Such vacuolar spaces would limit the mobility of these cells because of the increase of inertia and friction and interfere with the acquisition of material or for location of a mating cell.

#### **D. Structure and Function of the Cell Wall**

The cell wall of a plant cell forms an elastic corset, against which the protoplast is pressed by the turgor with great force. The cell walls, which are under tensile stress, have to fulfill paradoxical requirements with respect to their mechanical properties. Cell walls have to be tough and stiff to confer form and stability to the cell. On the other hand, cell walls must be able to grow. This means they must be expandable. The complex structure and changes during development accommodate these conflicting demands. It is striking that the basic cell wall architecture matches the structure of the main form-determining tissues of other organisms, where a fibrillar elastic substance is embedded into an amorphous plastic matrix such as the collagen of animals into a mucopolysaccharide matrix or the chitin of fungi and arthropods into a protein matrix. In plant cells the elastic microfibrils are embedded into a plastic matrix of pectins, hemicelluloses, and cell wall proteins.

The composition of the primary wall that is first formed during development is shown in Table 1. Here the proportion of cellulose is relatively low. The fibrillar structure of the cell wall results from cellulose molecules bundled to micelles, which again are bundled to micro- and macrofibrils (Fig. 12). During this process intermicellar and interfibrillar spaces are omitted as cavities. These are responsible for the permeability of the cell wall water and solutes, which therefore move in free spaces. The true barrier for the permeability of the cell is the plasmalemma.

Because of the large surfaces within these cavities the cell wall plays an important role in the absorption of solutes. Because of the negative charges of galacturonic acid residues within the pectin polysaccharids, mainly cations are bound and interchanged. These positions are available of course as first contact sites for a range of pollutants, which in this way may accumulate in large amounts.

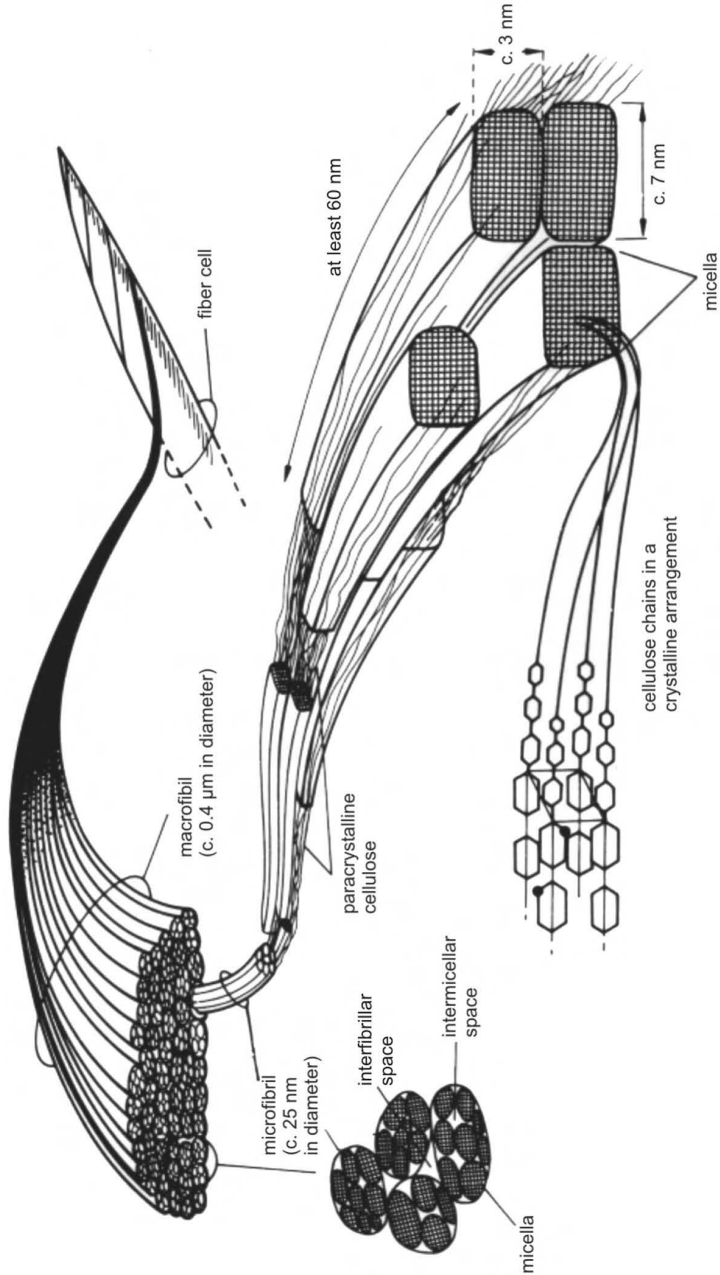
Wall properties depend on the proportion of the basic components of the cell wall. In the primary cell wall, which appears first during development, the proportion of cellulose is relatively small. In this stage

**Table 1** Composition of the Primary Cell Wall of Dicots and Grasses

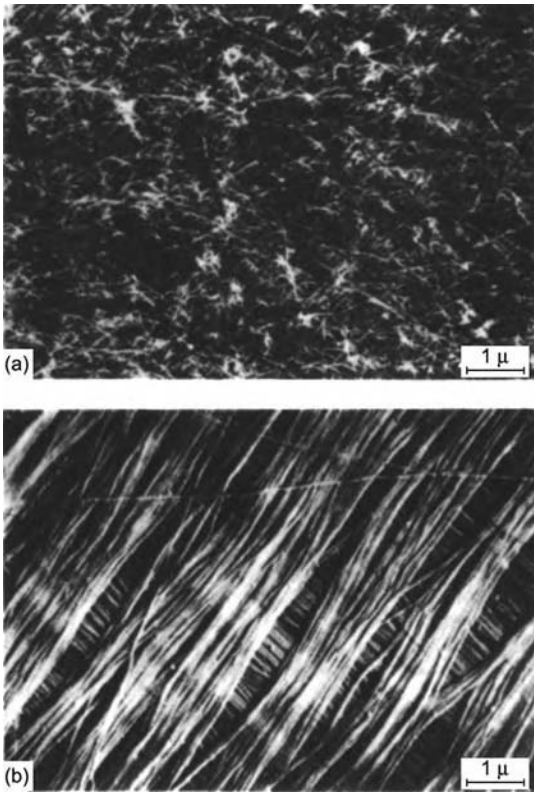
Polymer	Main components	Estimated proportion of the dry mass, %	
		Dicots	Grasses
<b>Cellulose</b>	1→4 $\beta$ -Glucose	20–40	20–40
<b>Hemicelluloses</b>			
Xyloglucan	1→4 $\beta$ -Glucose, $\alpha$ -xylose $\alpha$ -arabinose, $\beta$ -galactose, $\alpha$ -fructose	25	2–5
Xylane (heteroxylane)	1→4 $\beta$ -Xylose, $\alpha$ -arabinose, $\alpha$ -glucuronic acid	2–5	20–30
Callose	1→3 $\beta$ -Glucose		
Arabinogalactan	1→3, 1→6 $\beta$ -Galactose, arabinose		
1→3, 1→4 $\beta$ -D-Glucane	$\beta$ -Glucose	0	15–30
<b>Pectine</b>			
Polygalacturonic acid	$\alpha$ -Galacturonic acid	15	
Rhamnogalacturonan I	$\alpha$ -Galacturonic acid, $\alpha$ -rhamnose, $\beta$ -galactose, $\alpha$ -fructose	15	5
Rhamnogalacturonan II	$\alpha$ -Galacturonic acid, $\beta$ -rhamnose, $\beta$ -galacturonic acid, apiose, etc.	5	
Arabinan	$\alpha$ -Arabinose	low	?
Galactane	$\beta$ -Galactose	low	?
<b>Glycoproteins</b>			
HRGPs (hydroxyproline-rich glycoproteins)	Hydroxyproline, proline, lysine, serine, $\beta$ -arabinose, $\alpha$ -arabinose, $\alpha$ -galactose	5	0.5
Extensin	Repeat of serine- (hydroxyprolin)4 segments)		
PRPs (proline-rich glycoproteins)	Proline, valine, lysine Repeat of proline–proline– valine–x–lysine segments)	?	?
GRPs (glycine-rich proteins)	Glycine	?	?

Source: Adapted from Ref. 11.

the microfibrils are oriented within a planar sheet parallel to the plane of the plasma membrane according to the principle of dispersed texture (Fig. 13a). In this case the fibrillar structure can be easily displaced—a crucial requirement for cell enlargement. In addition to the random orientation, which can be recognized in the *Valonia* siphonal green algae (Fig. 13a),



**Figure 12** Arrangement of cellulose in the cell wall. (From Ref. 12.)

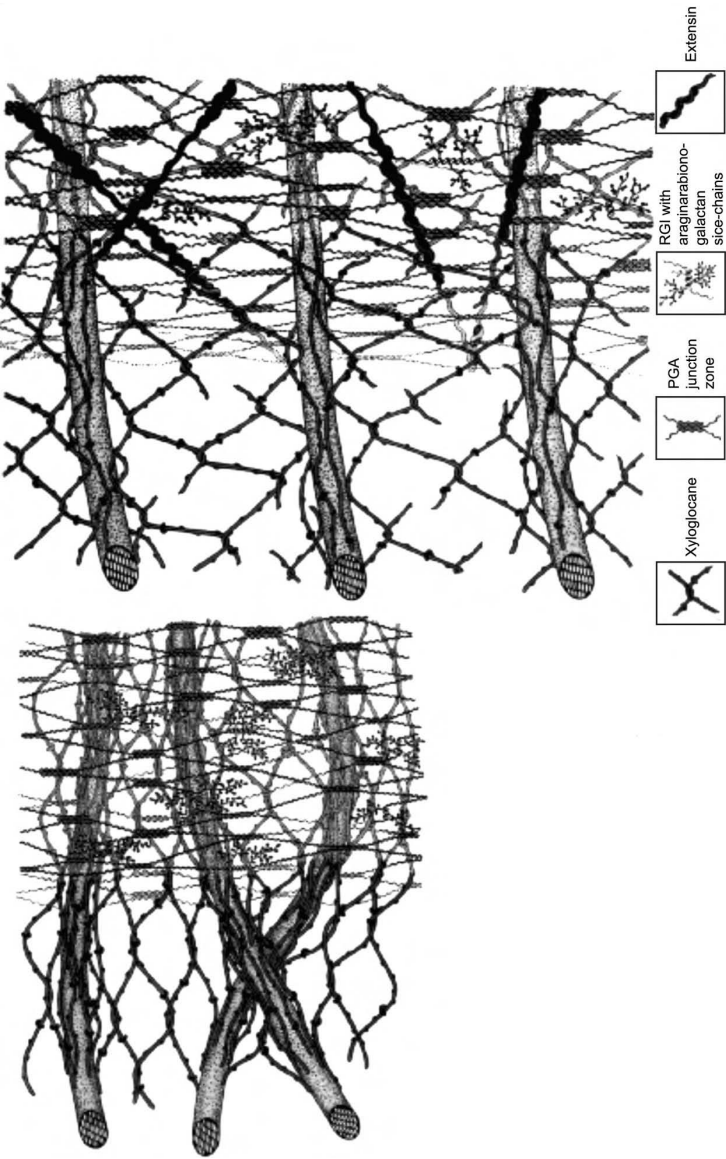


**Figure 13** Cell wall of the *Alga Valonia* (13): a, Primary wall with disperse texture; b, secondary wall with parallel texture, arranged in layers.

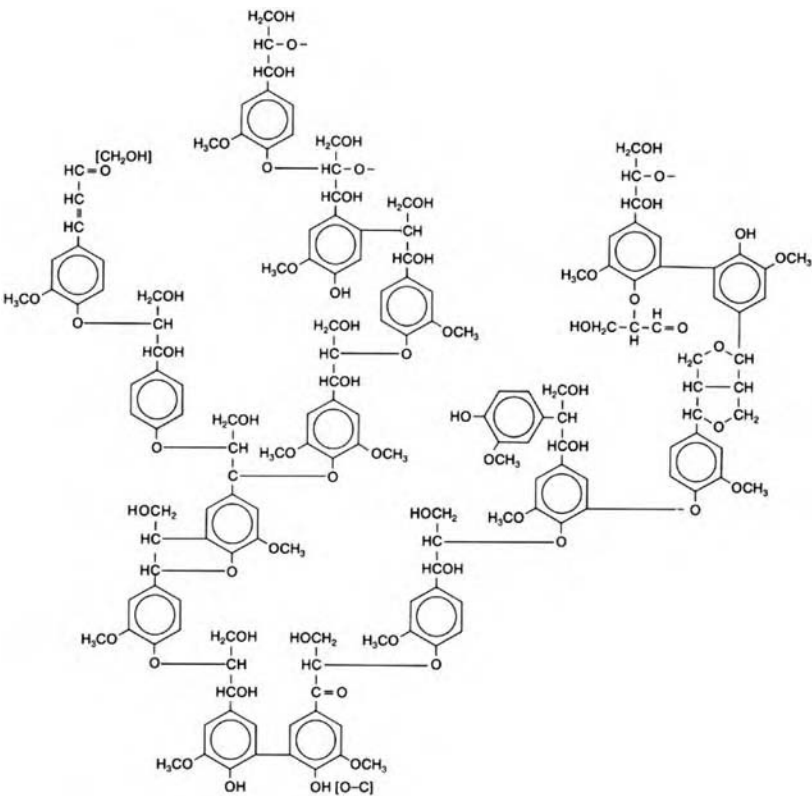
there are variants with a preferential direction of microfibrils, e.g., in multilayered and helicoidal walls. Embryonic cells, meristematic tissues, but also collenchymas and several parenchymas remain in the stage of the primary wall. After terminating cell growth many cell types produce a complex secondary wall below the primary wall. The microfibrils of the secondary wall are arranged more densely according to the principle of parallel texture (Fig. 13b). The proportion of cellulose is above 60%. The wall is elastically extensible but not capable of elongation growth.

Cell elongation results from an increase of the extensibility of the cell wall, but not an increase of turgor pressure. The model in Fig. 14 assumes the existence of independent polymer networks (a hemicellulose–cellulose fibril network, a pectin network, and a protein network) which after





**Figure 14** Extension growth of the primary wall of type I (14). The microfibrils of a single layer are arranged in parallel in a helical assembly around the expanding cell. They are held together by a network of hemicellulose (xyloglucan) chains, which are embedded into a matrix of pectins with the main components polygalacturonic acid (PGA) and rhamnogalacturan I (RGI). The comparison before (left) and after (right) the extension growth explains the relevance of xyloglucan splitting to the extension of the cell wall. The role of extensin synthesis for fixing the final cell form is also illustrated.



**Figure 15** Model of lignin structure.

termination of cell elongation determine the form. As an example, type I, which is the most common type in seed plants, is shown. It differs from type II, of grasses, mainly in its hemicellulose composition and its  $\beta$ -D-glucan synthesis during elongation (14). Figure 14 illustrates that the xyloglucans serve as cross-bridges and carry the main load in the longitudinal direction of stretching cells. In this case the microfibrils are helically arranged around the cells. Cleaving or dissociation of xyloglucans leads to a loosening of the microfibrils, which then separate in the direction of the longitudinal axis. Pectins with polygalacturonic acid (PGA) and rhamnogalacturonan I (RG I) as main components are assumed to play an important role in the control of xyloglucan cleaving. But it is still under discussion how the increase of elongation growth by phytohormones such as auxin takes place. Hypotheses include an acidification of the cell wall followed by a breakage of acid-labile load-bearing bonds (acid growth theory), a specific

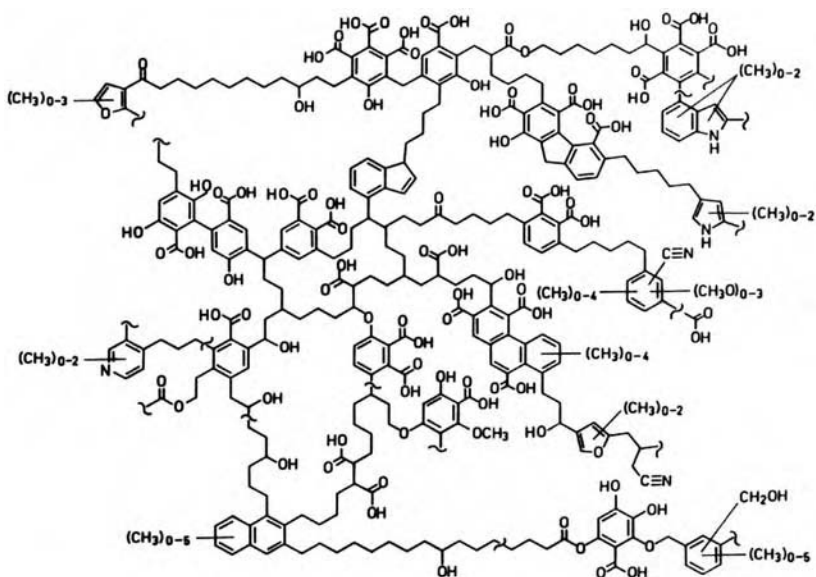


degradation of certain cell wall polymers, as well as the control of extensibility by the biosynthesis of growth-limiting proteins (11).

The dynamic properties of the cell wall play a crucial role in the control of phytopathogenic organisms. The cell has several possibilities to adjust its wall composition. In this case incrustation of substances (e.g., lignin, tanning derivatives, and minerals) has to be distinguished from accrustation of substances (e.g., cutin, suberin).

Lignin (Fig. 15) is second to cellulose, the quantitatively most important macromolecule on Earth. Its "invention" belongs to the most crucial steps in the evolution of higher plants as land plants. Its inclusion as a structural substance in the cell wall leading to lignification is responsible for the total impermeability as well as the hardness and compressive strength. In addition lignin plays an important role as a protection measure against infection and wounds. Lignin forms a complicated three-dimensional network, composed of phenylpropane derivatives. The attachment to cell wall hemicelluloses and cellulose is not yet completely understood.

Further degradation of plant detritus, with large amounts of lignin, as well as animal remains, leads to the formation of highly polymeric and stable humic substances, which accumulate in soil and contribute together with clay minerals to soil capability for ion exchange. Figure 16 shows a structural model of humic acids.



**Figure 16** Model of the macromolecular humic acid structure. (From Ref. 15.)

Cutin and suberin have a fundamental role similar to that of lignin in plants. They are true polyester compounds originating from lipid metabolism and have important functions as boundary layers and diffusion barriers, particularly for polar substances (e.g., water and ions). Both polymers occur together with complex mixtures of relatively unpolar lipids. Because of their beeswaxlike physical properties they are collectively called *waxes*.

Cutin is a component of the cuticular lamella on the outer epidermal walls of shoots, along with an additional layer, the proper cuticle, a 1- to 15- $\mu\text{m}$ -thick lamella, which is lying on the cuticular lamella and often overlain by an epicuticular wax layer, usually in the form of wax aggregates protruding above the surface. In addition intracuticular waxes are formed.

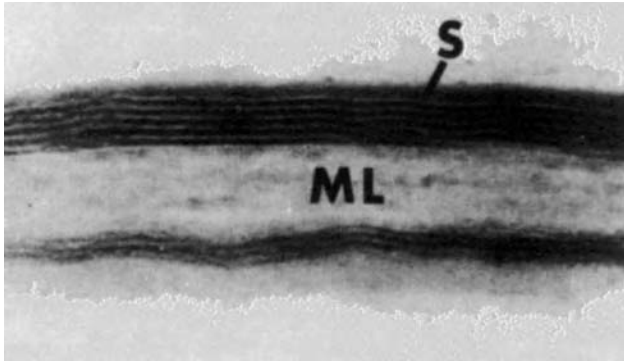
This construction creates a biological barrier to the airspace. It not only provides protection against water loss (by transpiration) or leaking of ions (by rain) or, in the opposite direction, a diffusion barrier for water and solutes (among them agricultural chemicals and pollutants), but also offers protection against phytopathogenic organisms.

The major role of epicuticular wax aggregates is as a transport-limiting barrier, which forms a frostlike layer and creates a hydrophobic and microscopically rough leaf surface, which is not wettable by water. This structure leads to a spherical rounding of water droplets (Fig. 17) and prevents leaking of ions and other substances from the leaves.

The polymer is composed of two groups of hydroxy and epoxy fatty acids with chain lengths of  $\text{C}_{16}$  and  $\text{C}_{18}$ . In addition there are smaller amounts of phenolic acids, esterified with cutin. Cutinase attack by phytopathogenic fungi releases phenolic acids, which have a toxic effect on the pathogens. Covalent linking of pesticides with the cuticular polyester structure opens the possibility for optimal use of these compounds: only an attack by phytopathogenic organisms releases the bioactive compound.



**Figure 17** Water droplet on a *Brassica* leaf. (From Ref. 16.)



**Figure 18** Suberin (S) from the periderm of a potato tuber. ML, middle lamella. (From Ref. 17.)

This means that the effectiveness and protection would occur at the right time with a minimum of applied amounts.

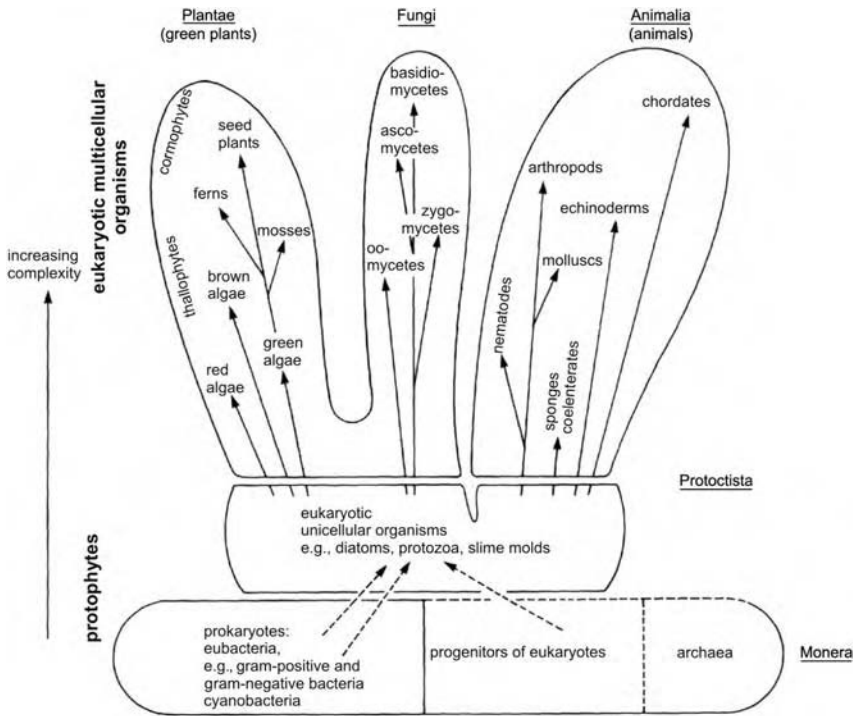
Suberin together with waxes forms an important component of cork cells. The layered lamellae occur especially in wound tissues, periderm, as well as inner dermal tissues such as the endodermis or the bundle sheaths of grasses. They are layered from the inside onto the secondary wall as distinct layers (Fig. 18). Frequently a lignified cellulose layer is added.

The suberized cells die and form a compact and totally impermeable isolation and protection layer with functions comparable to those of the cuticle. The chemical composition differs from that of cutin in a higher amount of phenolic compounds and  $\omega$ -hydroxy acids. In addition the chain length of the respective dicarboxylic acids, fatty acids, and alcohols often exceeds 18. In contrast, epoxy and polyhydroxy acids are less frequent.

### III. DIFFERENT LEVELS OF ORGANIZATION IN PLANTS

The cell as smallest autonomous unit of all living systems is a clearly defined entity with the same basic configuration and pattern of physiological reactions throughout the plant body. However, cells constitute extremely complex systems with regard to their coordination within multicellular organisms. As a result of cooperative and competitive interactions they display unexpected “new” properties, the *system properties*.

In the plant kingdom there is a variety of levels of organization (Fig. 19), which represent various strategies for adaptation to the environment. In the most simple case, the entire organism corresponds to a single cell



**Figure 19** Plant organizational levels and their phylogenetic origin. For comparison the taxonomic classification (five kingdoms (18)) is underlined. Within the group of protocists there is no matching with the grouping into organizational levels.

(protophytic level). Many examples are found within the group of algae, but also among fungi.

This was the basis for the integration of cells to more complex units during the course of evolution, which reached the highest level with true multicellular organisms. In this case the different cells arise by division. The multicellular forms in the plant kingdom can be assigned to two levels of organization, the thallophytes, which are mainly adapted to life in water, and the more complex cormophytes, which are perfectly adapted to life on land.

The thallophytes, also called *lower plants*, include many algae and most of the fungi. They have a filamentous or two-dimensional body, which exhibits no or, with the exception of several brown algae, little differentiation in their vegetative part. Important examples are the filamentous and derived lichen thallus as well as the tissue thallus.

**Table 2** Taxonomy of Higher Plants, Simplified

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Phylum Pteridophyta (ferns and their relatives)
Class Pteridopsida (ferns)
Class Lycopodiopsida (club mosses)
Class Equisetopsida (horse tails)
Phylum Spermatophyta (seed plants)
Subphylum Gymnospermae (gymnosperms) <sup>a</sup>
Subphylum Angiospermae (angiosperms) <sup>a</sup>
Class Dicotyledoneae (dicots)
Class Monocotyledoneae (monocots)

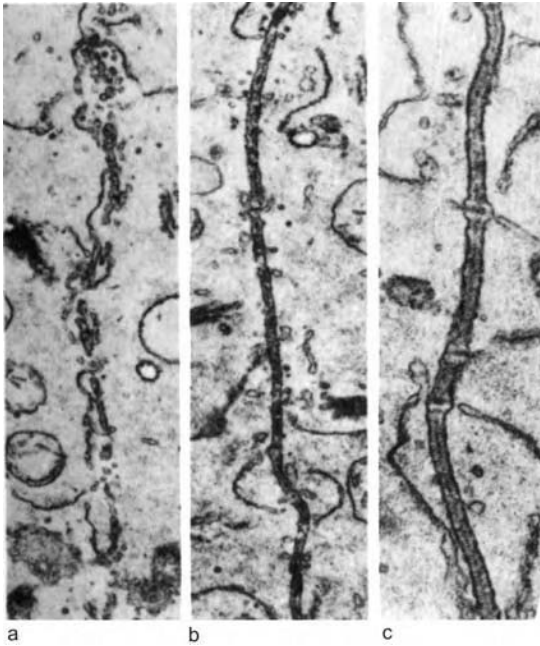
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<sup>a</sup>A more detailed classification is not considered here.

In contrast, the kormophytes (higher plants) exhibit a clear organization with the basic organs shoot and root, which does not occur in any other group. Kormophytes include the lower and higher vascular plants (Table 2). The latter comprise the gymno- and angiosperms and represent the largest and most important group of the plant kingdom. They represent the major part of all plants on earth, most of the wild and cultivated plants belong to them. With regard to their organizational level mosses take an intermediate position between thallophytes and kormophytes.

### A. Tissues of the Higher Plant

The structure of plant organs clearly shows the integration of cells of the same function in collectives, called *tissues*. Within this organization the cells abandon their independence. Instead certain common functions are performed, which are characteristic of the respective type of tissue and crucial to preservation of life by the entire organism. The tight functional integration of cells within a tissue is a result of common origin from a single mother cell. During cell division the daughter cells do not completely separate but remain connected by plasma strands (plasmodesms), which penetrate the cell wall (Fig. 20). Areas where plasmodesms are clustered can be recognized as pits (Fig. 4a). In this region the secondary cell walls are omitted. The connections of cells by plasmodesms result in a linkage of all protoplasts, at least within a tissue, to a joint unit, the symplast. Within this space a short-distance transport of solutes and therefore also pollutants takes place. The extraprotoplasmic space, which includes the cell walls as well as the open spaces between the cells (intercellular spaces), is the apoplast. Also in this case there is a continuum where the solutes can move within the cell walls far inside the tissues. Transport speed is mainly



**Figure 20** Formation of the cell wall and origin of plasmodesms (19): a, Fusion of dicytosomes into the cell plate; b, early stage of the primary cell wall; c, fully differentiated primary wall with plasmodesms.

determined by diffusion. In addition, the intercellular spaces play an important role in the distribution of gaseous pollutants in the plants.

Different types of tissues can be discriminated according to the multitude of functions. In addition to simple tissues, which are composed of one cell type, there are complex tissues formed from several cell types, which serve a cross-functional purpose. The complex achievements of higher organisms, which are mainly adapted to the environment on land, are only possible because of the grouping of cells to tissues and, simultaneous division of labor at the level of tissues.

The organization of plant tissues is characterized by a division into tissues with embryonic cells (meristems) and tissues with differentiated cells (primary and secondary tissues). In contrast to the organization of animal tissues, this division prevails for the entire lifetime of the plant organism. The task of meristematic cells is the production of daughter cells, most of them develop to primary and secondary tissues. One of the important properties of meristematic cells is related to the property that at least one of the daughter cells maintains its embryonic properties. Meristematic cells are

usually more sensitive to pollutants than differentiated cells. This explains the high sensitivity of growth to toxic compounds.

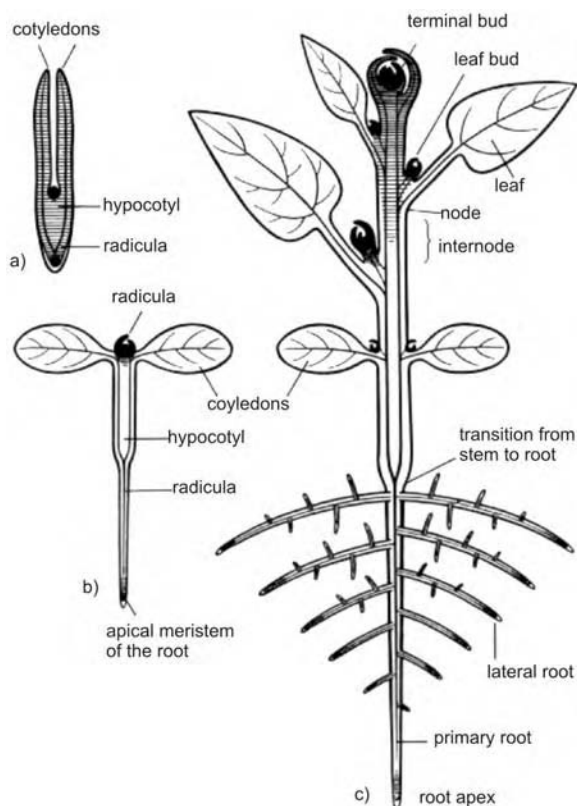
The apical meristems at the apex of the shoot and root are directly derived from embryonic cells. They produce primary tissues, which build the body of the herbaceous plant. After primary growth the plant can switch to a phase of secondary growth. Here large amounts of secondary vascular tissue but also periderm are produced. Secondary growth coincides with an increase in thickness, visible in many perennials, e.g., in most trees and shrubs. It is based on the activity of two meristem cylinders, the cambium and the cork cambium. The cambium is in most cases directly derived from the apical meristem, the cork cambium always indirectly, as it originates from differentiated cells.

## B. Plant Organs

The structure of the higher plants shows, despite their tremendous diversity, a common principle of organization, which was known by the name *kormus* (gr: stump) in the classical literature. The main feature is the subdivision of the vegetation organs into shoot (stem plus leaves) and root. Figure 21 schematically shows the characteristic structure of a dicot during its primary growth. The flower is a modified shoot, composed of a flower axis and modified leaves directly or indirectly involved in reproduction. The basic organs of the plant can already be recognized in the embryo (Fig. 21a). They are called cotyledons, hypocotyls, and radicles. In contrast, the adult plant (Fig. 21c) is usually composed of several organs, to which flowers are added later as further organ systems. This means that the production of organs is not terminated in the embryonic stage, as is the case with animals, but continues throughout the entire lifetime, even in the stage of the adult plant. The meristems in the shoot and root apices are responsible for this feature. These tissues are the most sensitive structures of plants. Exposure to pollutants leads to early damage.

The fundamental differences between plants and animals include the characteristic that in animals there is already in early embryonic stages a separation into germline and somatic cells. This is not the case in plants. The germline cells of animals differentiate during their development to male and female germ cells. In contrast, the somatic cells form the organs including the gonads. The stronger determinate development of animals differs particularly in its special pattern of gene expression during embryo development, which has been referred to as *zootype* (21) and differs from that of plants and fungi, whose development is more plastic. Plant germ cells cannot be traced back to early stages of embryo development. They only differentiate in later developmental stages.





**Figure 21** Organization of the plant body using a dicot as an example (20): a, embryo; b, seedling; c, plant during primary growth.

During evolution of organisms a distinct trend to enlargement can be recognized. In plants it usually does not lead to the construction of more massive structures, but to dendritic structures. Branching is one of the most important processes during plant development. This process transforms the simple primary shoot into a shoot system and the primary root into a root system. In shoots new axes arise by sprouting of leaf buds, in roots by the growth of endogenously originating lateral root primordia. Continuous repetition of these processes can generate complicated branching systems.

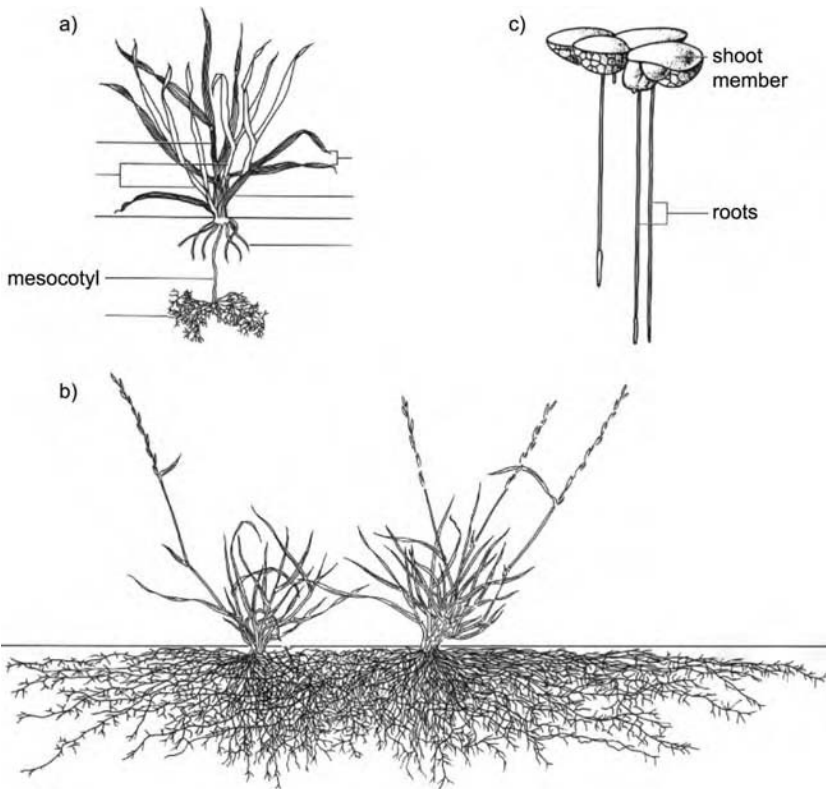
The laminar form of leaves, which are preferentially found in larger shoot systems at the ends of the dendritic structure, provides a large surface, useful for effective light absorption.

However, the optimal usage of the available space is particularly disadvantageous in a polluted environment. Therefore, plants are particularly



sensitive to those pollutants that can be taken up. The import across a huge surface and the lack of an effective excretion system endanger plants, which cannot change their position voluntarily, in a polluted environment. The forest dieback and the retreat of lichens illustrate this characteristic.

In seed plants there are many variants with respect to their morphological features. But all of them can be traced to the basic structure of the kormus. Two extreme examples of monocots' remaining throughout their entire life cycle in the phase of primary growth illustrate this phenomenon. At stems of grasses nodes where the leaves are attached can be easily recognized. Each leaf first encloses the stem in tubelike manner as sheath, before it extends to the leaf blade. An eyrie (Fig. 22a,b) develops when the buds in the leaf axils of the basal sheaths sprout to lateral axes.



**Figure 22** Variation of shoots: a, Structure of an eyrie. (From Ref. 22.) b, Growth of a lawn with *Lolium perenne* by tillers. (From Ref. 23.) c, Shoot member of the water lense *Lemna gibba*. (From Ref. 24.)

They either grow up within the surrounding sheath (dense eyrie) or penetrate the sheath, followed by an upraising after brief horizontal growth. Then a new stem of the same morphological structure is formed and generates a strong root system. The repeated process leads to the growth of a lawn because of the low position of buds. A cut does not affect growth but stimulates sprouting of new buds within the lowest sheaths. Particularly effective spreading is observed in those species in which the lateral shoots grow to above ground or underground stolons and again form a new eyrie within some distance of the mother plant (Fig. 22b). A counterexample is seen in water lenses, which swim at the surface of ponds and as an adaptation to their pattern of life have no distinct structuring of their shoots (Fig. 22c). The small, usually lenslike vegetation bodies consist of a thalluslike shoot member with a filamentous root and two lateral pockets, from which the daughter shoots arise. The species *Wolffia arrhiza* with a maximal length of 1.5 mm is the smallest seed plant.

### C. Structure of the Primary Stem

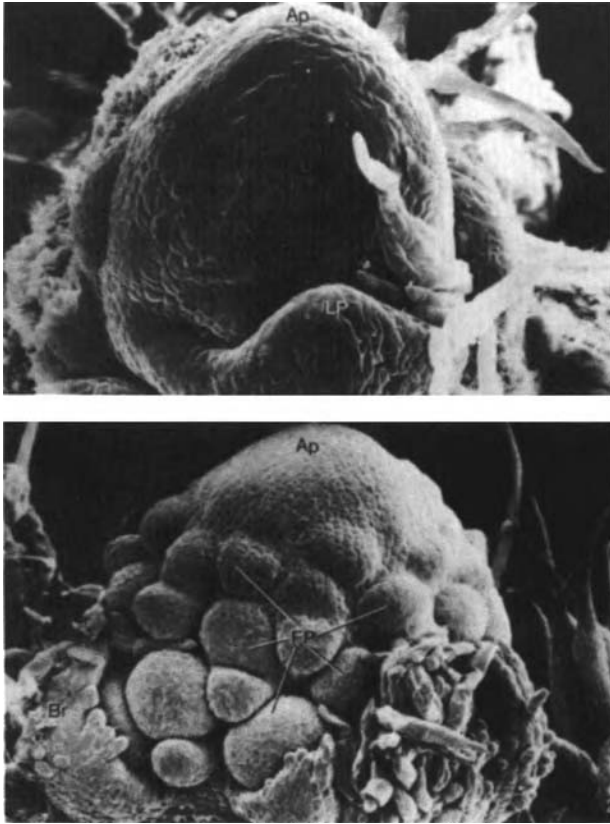
At the shoot apex the apical meristem is located, enclosed by the terminal bud (Fig. 23). The upper part of the apical dome, measuring only a fraction of a millimeter, is the apical meristem. Its descendants differentiate after a few further divisions and after termination of cell growth to the mature primary stem.

Disturbances of growth such as distortions or stunting, which in part can be traced to pollutants, but also to the influence of pathogenic organisms, are triggered by interference with the apical and subapical areas.

Directly below the apical meristem the leaf primordia can be recognized as small bulges. During further development they give rise to the formation of leaves. Therefore leaf position is already determined at the shoot apex. Because in most cases lateral buds develop in the leaf axils, the position of potential lateral shoots is also determined early. The structures within the leaf buds do not differ from those of the terminal bud.

One of the most important stages in the development of a higher plant is the transition from the vegetative to the reproductive phase, which is recognized first at the shoot apex. Instead of leaf primordia (Fig. 23a) flower primordia (Fig. 23b) are now produced. With their further development to flowers or inflorescences the shoot axes terminate growth at this position after seed and fruit formation.

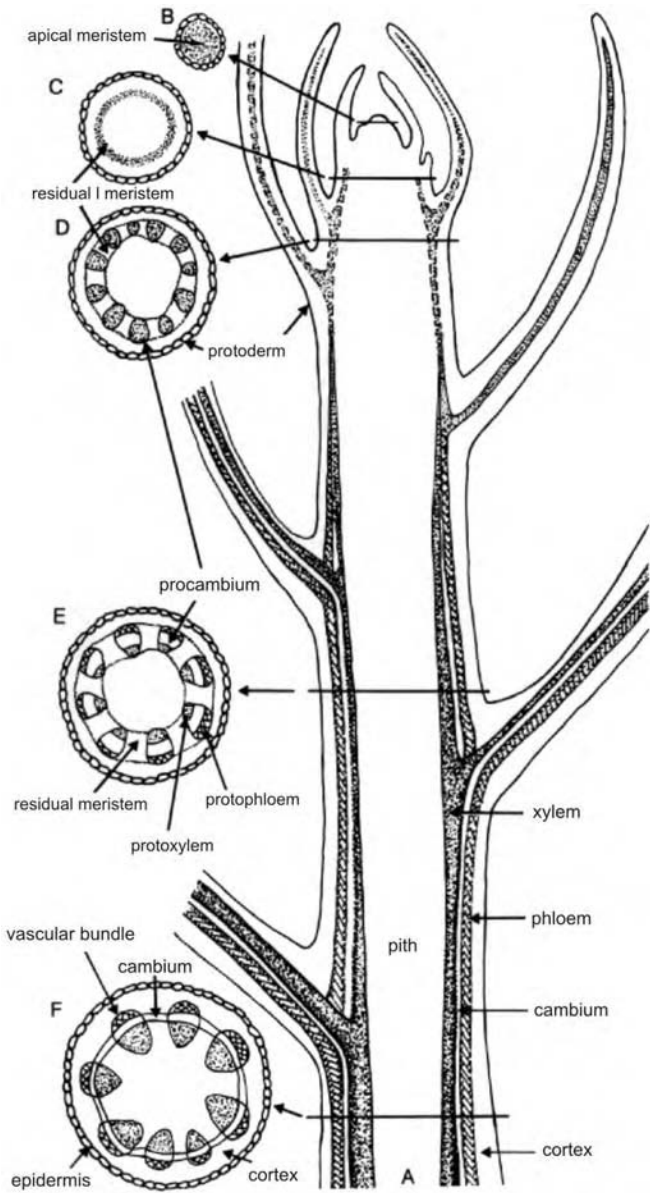
An increase in flower and fruit formation does not necessarily indicate an optimal physiological process. It is frequently a sign of an “emergency situation” under various stress conditions. A typical example is the excessive cone formation in diseased firs and spruces.



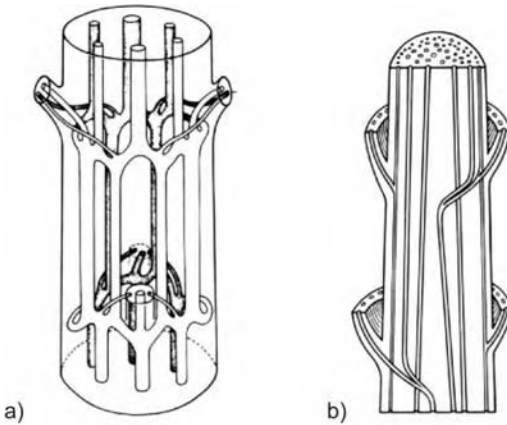
**Figure 23** Shoot apex of *Xanthium strumarium* (25): 1, Vegetative stage (above); 2, reproductive stage (below). Ap, apical meristem; LP, leaf primordium; BR, bract; FP, flowering primordia of the staminate (male) inflorescence.

The development of the cells derived from the apical meristem to their final form and function within the stem can be reconstructed by serial cuts. Figure 24 shows the most important developmental stages of a dicot. The main tissues of the primary stem are the epidermis as a surface layer of cells covering the primary tissues, the parenchyma as ground tissue of the cortex and pith, and the vascular tissues found within the vascular bundles. The differences in the primary structure of the stem of different plant groups are mainly based on differences in the relative distribution of parenchyma and vascular tissues. In addition mechanical and secretory tissues can occur.

The distribution of tissues within the stem is mainly determined by biomechanical requirements. Even in lightweight construction, requiring



**Figure 24** Scheme of a longitudinal cut (A) through the shoot apex of a dicot and cross sections (B-F) of different developmental stages. (From Ref. 26.)



**Figure 25** Arrangement of vascular bundles: a, dicot; b, monocot. (From Ref. 27.)

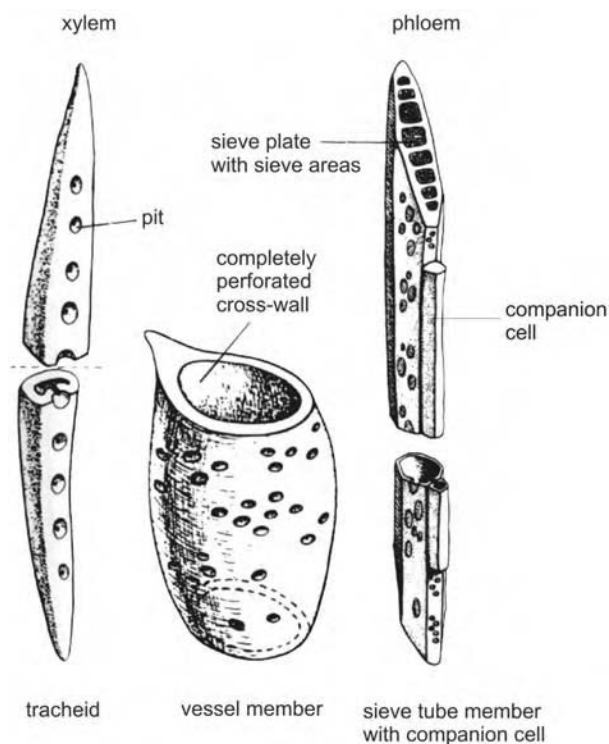
little material employment, an optimal arrangement of the supporting elements favors resistance to static and dynamic loads. In contrast to technical constructions the plant does not require complex and stiff construction. In this case we usually find elastic structures, which oscillate during wind impact without buckling or tearing.

Whereas the vascular bundle system in gymnosperms and most dicots is restricted to a hollow cylinder separating pith from cortex, ferns and monocots have a more complex arrangement. Figure 25 shows the most frequent constructs.

The vascular tissues within the vascular bundle are characterized by their complex structure built from two entirely different components. The xylem, where mainly water and dissolved mineral salts are transported, contains as functional elements the tracheids and vessel elements (Fig. 26). In both cell types the protoplast is eliminated in the functional stage. The required stability is provided by strong, unevenly thickened cell walls that usually contain large amounts of lignin. In the vessel members the cross-walls are partly or totally removed, providing long continuous vessels.

The phloem transports assimilates. It is the pipeline of the plant. The sieve elements consist of living cells in their functional stage. Whereas in ferns and gymnosperms the simpler sieve cells are found, angiosperms have sieve-tube members, which are always accompanied by companion cells (Fig. 26). The principal morphological characteristics are the absence of nuclei and tonoplasts in fully developed sieve elements. In addition the cross walls and parts of the longitudinal walls are perforated to form sieve plates.

The relevance of the conducting elements to the transport of pollutants is discussed in Chapter 3. The impairment of assimilate flow



**Figure 26** Conducting elements of the xylem and the phloem. (From Ref. 28.)

interferes with vital functions of the plants. On the other hand, the vascular tissues can be used for the distribution of phytopharmacological compounds, for instance, systemic fungicides.

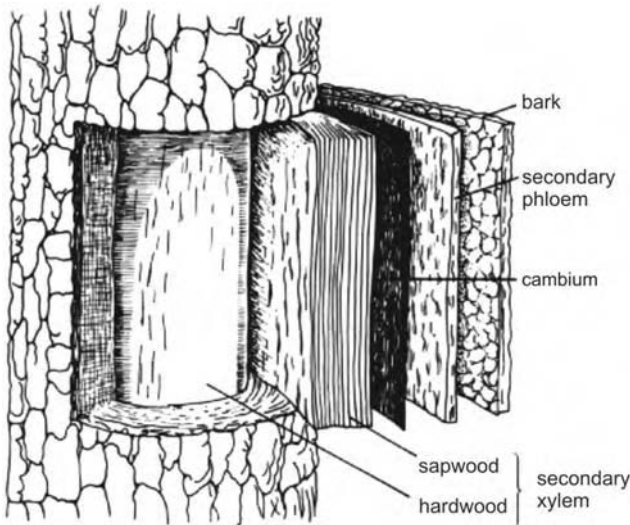
#### **D. Structure of the Secondary Stem**

In most gymnosperms and dicots the tissues of the primary stem are only sufficient for the static requirements of the first vegetation period. Therefore additional tissues have to be formed during further growth. This growth leads to an increase of the circumference of the stem and mainly provides secondary vascular tissues that also contribute to an increase of stability. Secondary growth starts only in those parts of the stem that have completed their primary growth, i.e., arrangement of the basic pattern of tissues and longitudinal growth. This construction provides a continuous connection of living cells between newly formed primary tissues of developing shoots and roots. It also allows the longevity of a plant although individual cells

usually reach only a maximal age of 3 years. Extreme examples of long-living trees are *Pinus aristata* (4600 years), linden (1900 years), spruce (1100 years), pine, fir and elm (c. 500 years). These numbers are of course not identical with the rotation period of these trees.

The activity of the vascular cambium contributes to the main part of secondary growth (Fig. 27). It is a cylindrical meristem, which in most trees already exists in the primary stem as a closed cylinder between primary xylem and primary phloem. The cambium forms inward the secondary xylem (= wood) and outward the secondary phloem (= bast). The production of wood accounts for most of the secondary growth. The annual rhythm of cambium activity starts in spring with the production of wide wood elements. During the vegetation period it gives way to the production of narrower cells. Therefore annual rings can be seen with the naked eye.

Wood of gymnosperms can be recognized by its more uniform and simple structure when compared to dicot wood. However, in both cases the main functions are the transport and storage of water and mineral salts, the maintenance of stability, and the storage of assimilates. Several tree diseases are characterized by typical damage of wood. This includes the wet core, which plays a role in fir but also in several broadleaf trees. Another common type of damage is wood rot, which is caused by lignin-degrading fungi (e.g., *Phanerochaete*).



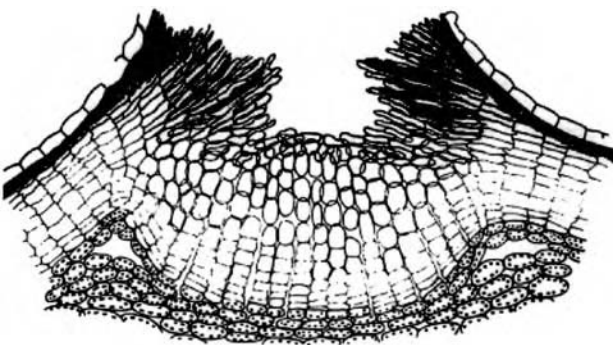
**Figure 27** Structure of a pine stem.



The small part of secondary phloem within the secondary stem makes recognition of its role as the pipeline of the plant difficult: its main function is the conduction of assimilates. In addition the storage of assimilates is important. Both take place in the soft part of the phloem including sieve elements and parenchyma cells. In contrast fibers, often found in small groups, provide stability and tensile strength. The alternation of these two components of the secondary phloem accounts for multiple layering within an annual increment.

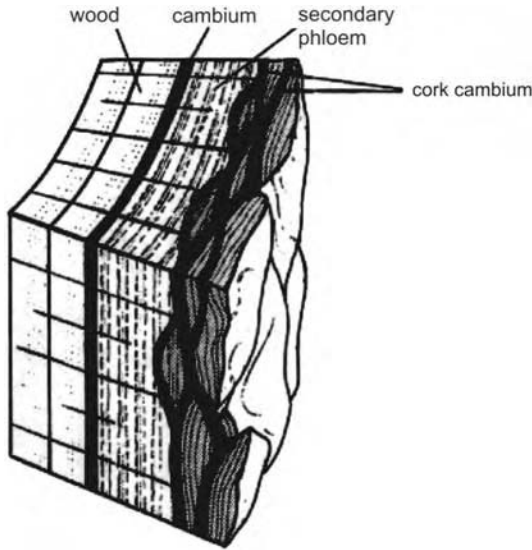
Because of the secondary growth all tissues outside the cambium are stretched. This effect becomes stronger with increasing distance from the cambium. In order to prevent tensions at least within the current vegetation period, cell divisions take place in the living cells. They contribute to circumferential growth (dilatation growth). However, in the peripheral regions, particularly in the epidermis, cells are ruptured. Nevertheless, the stem can provide the necessary protection. Long before the onset of mechanical strain in of the epidermis the cork cambium (phellogen) is formed at the periphery of the stem. The cork cambium is a secondary meristem, which is responsible for the development of the periderm, a secondary dermal tissue. The outer descendants of the cork cambium develop into cork cells and are crucial for the function as a protective layer to prevent loss of water and injuries. All cells lying outside the cork eventually die. Gas exchange is only possible through the lenticels, masses of loose cells protruding above the surface through a rupture of the periderm. They contain large intercellular spaces (Fig. 28). On the other hand they may provide entry for phytopathogenic organisms.

In most cases new cork cambia develop in deeper layers with increasing age of the stem under the influence of the peripheral tension. Again this cork



**Figure 28** Cork layer with a lenticel from a *Sambucus* twig (cross section). (From Ref. 29.)





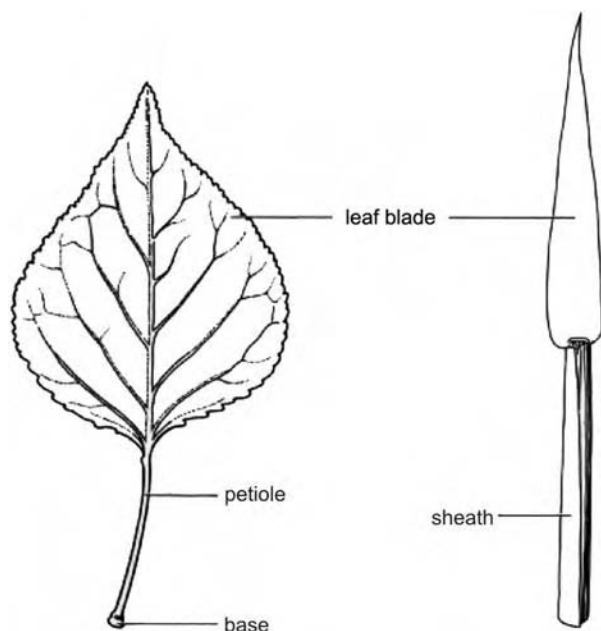
**Figure 29** Structure of the scale bark. (From Ref. 27.)

cambium forms its own layer of cork. A continuous repetition of these processes forms the bark, an accumulation of cork layers and dead peripheral tissues. During continued bark formation cork cambia are finally produced in the secondary phloem. Figure 29 shows the structure of the frequently occurring scale bark. The bark forms a highly efficient protection and isolation layer. Since the peripheral layers are continuously peeled off, substances taken up by the plant are eventually returned to the soil.

### **E. Structure and Function of the Leaf**

Leaves are lateral organs of the stem. Foliage leaves are the most important ones because of their functions in photosynthesis and transpiration. Figure 30 shows the structure of two common forms of foliage leaves.

The blade with its laminar form plays an important role in the functions mentioned. In contrast to growth of the stem growth of leaves is usually restricted. Their meristems are diffusely distributed and completely consumed during development. The anatomical structure of the blade, which normally does not show any secondary growth, is explained in Fig. 31. The main part of leaf tissue is composed of mesophyll, a parenchyma with many chloroplasts and a large volume of intercellular spaces. For many plants layering into a palisade parenchyma and spongy parenchyma is characteristic.

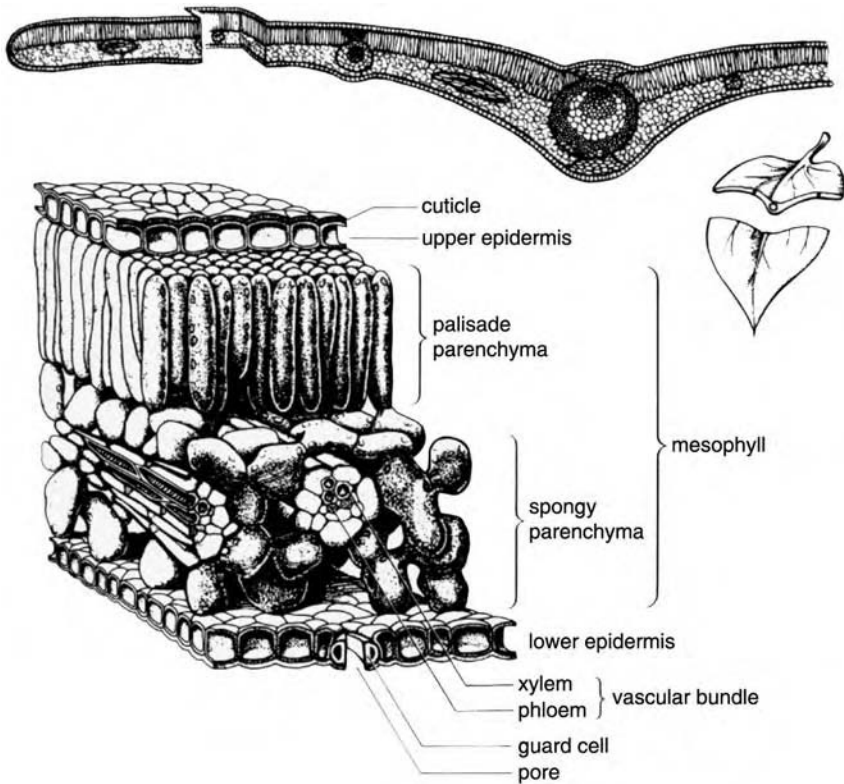


**Figure 30** Organization of a blade. Examples are a dicot (*Prunus* species) and a monocot (*Arundo donax*) leaf.

The vascular system, which can be recognized as veins, transports water, mineral salts, and organic nutrients. It is found in the upper part of the spongy parenchyma and is completely enclosed, even at its free ends, by an intercellular-free bundle sheath parenchyma.

The epidermis covers the entire leaf surface. It is a compact cell layer free of intercellular spaces. It completely encloses the mesophyll and is continuous with the surface of the stem. The cutinized cell walls and the coating by a cuticle provide effective protection against transpiration. The different layers of the outer wall have different affinities for applied bioactive compounds, e.g., fungicides. This characteristic is most important for an effective defense against pathogens.

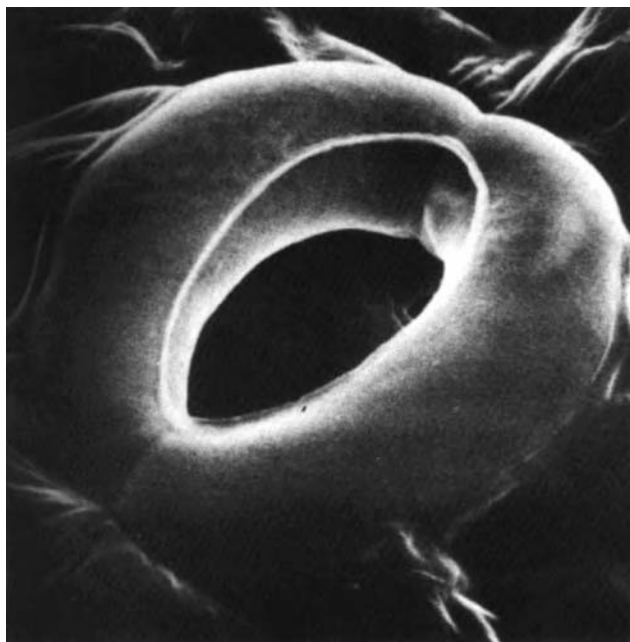
Gas exchange and therefore transpiration are mediated by the stomata, which are evenly distributed in the epidermis. Figure 32 explains the structure: two guard cells with a kidney shape give way to a pore in their turgid state. A decrease of the turgor leads to the closure of the pore. This opening and closing mechanism is controlled by a complex interaction of environmental factors (light, temperature,  $\text{CO}_2$  concentration, air moisture) and endogenous factors such as the concentration of abscisic



**Figure 31** Structure of the leaf blade. (From Ref. 28.)

acid. Interference by xenobiotics leads to severe damage, this includes changes in the wax particles, which in the young conifer needle fill the epistomatal space above the stomatal cavity as thin wax tubules (Fig. 33). Pollutants such as ozone and acid rain cause and accelerate aggregation of wax as well as formation of cracks (31). This process leads to the impairment of the natural opening and closing mechanisms as well as diminished protection from pathogens or penetration of aerosol particles.

A remarkable achievement of plants is their ability to meet their carbon requirement in the atmosphere with a  $\text{CO}_2$  concentration of only 0.03 vol% without risking a water deficit by transpiration. An essential prerequisite is that the reduction of stomatal conduction by partial stomatal closure more strongly interferes with transpiration than with assimilation (32). Under constant conditions (relative humidity, 55%, quantum flux,  $1.5 \text{ mmol/m}^2\text{s}$ ) leaves of *Xanthium strumarium* lose 336 molecules of water



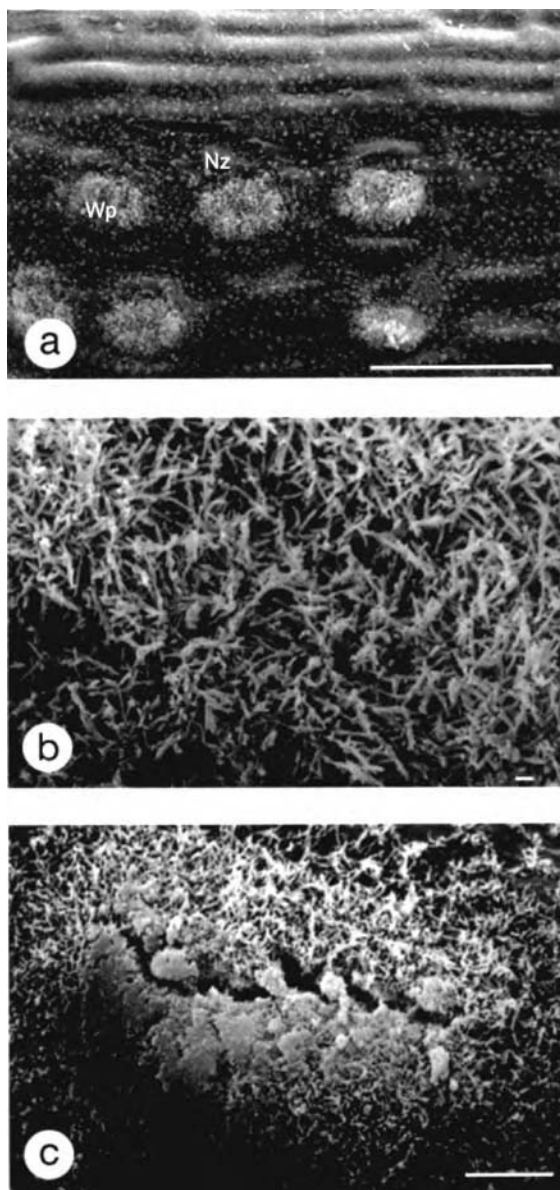
**Figure 32** Stoma of a cucumber leaf. (From Ref. 30.)

for each assimilated  $\text{CO}_2$  molecule at a stomatal conductance of  $0.4 \text{ mmol/m}^2\text{s}$ . If it is lowered to  $0.1 \text{ mmol/m}^2\text{s}$ , the ratio is decreased to 156 molecules of  $\text{H}_2\text{O}$  per assimilated molecule of  $\text{CO}_2$ .

During opening of the stomata the already considerable leaf surface increases by the surface area of the mesophyll cells adjacent to the stomata. This leads to an approximately five-fold increase of the area, which provides optimal contact to the outer atmosphere and thus favors  $\text{CO}_2$  absorption. But stomata are also ideal entries for pollutants. A considerable part of damage caused by environmental factors but also by infections of phytopathogenic organisms (bacteria and fungi) is due to this route.

The light conducting structures of leaves are remarkable (33). Spectacular are the lens effects of convex epidermal cells in shade plants, which have a higher refraction index than air. When light penetrates farther into the leaf tissue, scattering occurs. Reflective surfaces at the boundary layers from the epidermis to the air prevent light from escaping the leaf. After removal of the lower epidermis up to 15% of the light entering the leaf is lost.

Whereas light scattering in the upper mesophyll part and the reflecting boundary layers causes high fluorescent rates (light trap effect!), further



**Figure 33** Wax plug in young spruce needles: (31). a, Overview (reference bar 100 µm); b, detail (reference bar 1 µm) of the control; c, combined treatment with ozone and acidic fog (reference bar 10 µm). Wp, wax plug; Nz, lateral cells.

penetration of light leads to a rapid decrease of intensity. The light gradient enables the plant to determine light direction. In a spinach leaf up to 90% of the blue and red wavelengths are absorbed within the first 140  $\mu\text{m}$  of the mesophyll. This corresponds to the passage through two palisade cells. Less than 10% of the total light absorption is left for the sponge parenchyma. Therefore the distribution of photosynthetically active tissues over the cross section of the leaf is limited. Adaptations to different light intensities are sun leaves with their conspicuous palisade parenchyma cells and the much thinner shade leaves. Because of the special structure of palisade cells parallel light from direct sun irradiation can penetrate much deeper into the leaf than diffuse light in the case of shading.

Form and physiological characteristics of the remarkably plastic leaf organs are closely related to the respective ecological conditions. In this case, light, humidity, nutrition, and temperature play the main roles. Heat emission is particularly important for the energy balance of leaves. It depends on the wind speed, size and form of the leaf, curvature, and surface structure. Different combinations of environmental factors require different optimal solutions, which are represented in the multitude of leaf forms and structure.

Especially important are biochemical variants, which use different strategies for  $\text{CO}_2$  uptake. In this context  $\text{C}_4$  plants (e.g., corn, sugar cane, sorghum) provide high  $\text{CO}_2$  concentrations in those places where the Calvin cycle operates (bundle sheath cells) and therefore repress photorespiration. This is possible through effective prefixation of  $\text{CO}_2$  by phosphoenolpyruvate carboxylase in the mesophyll cells. The  $\text{C}_4$  oxalacetic acid is produced and is reduced to malic acid. The product is moved to the bundle sheath cells, where oxidation and decarboxylation take place.

The condition and appearance of leaf blades are important criteria for the evaluation of the general condition of a plant. Damage of various kinds is recognized by the formation of water spots, discoloration, yellowing, or necroses.

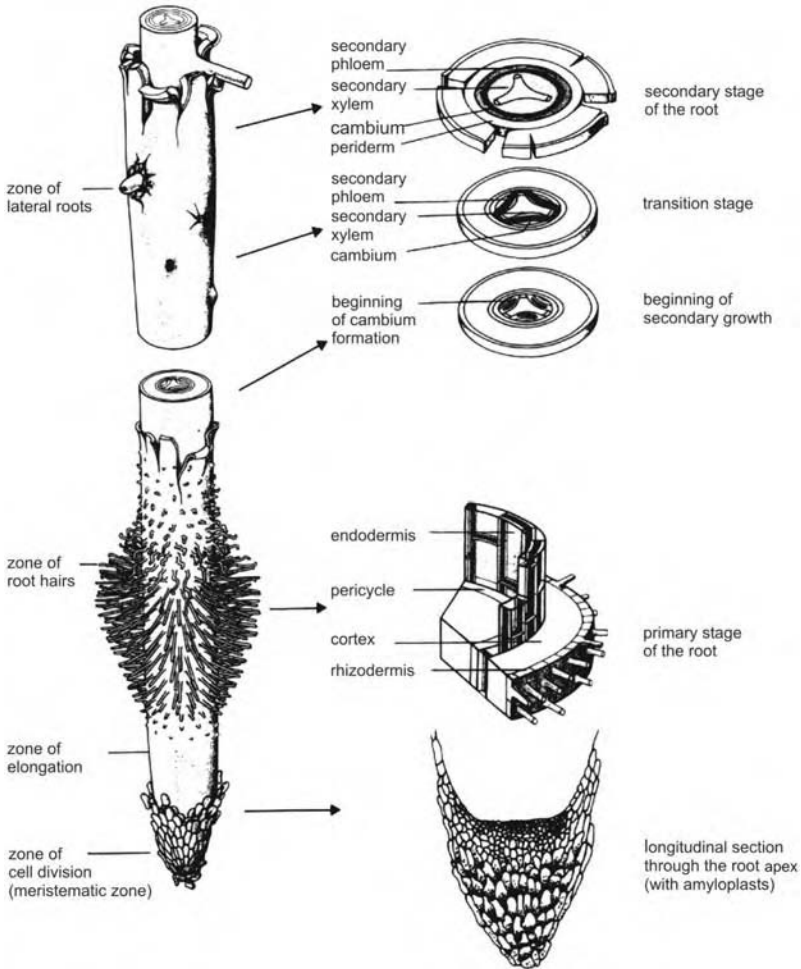
Plants with annual leaf shedding, which is anticipated in fall by the formation of a separation layer at the base of the petiole, avoid major accumulation of pollutants in the leaves. This problem is much more severe in evergreen leaves, which remain functional through several vegetation periods. This problem affects the needlelike leaves of gymnosperms with their xerophytic adaptations. They are shed in pines after 3 years or later, in spruce or fir only after 6 or 12 years, respectively. There is a close relation between the extended functioning of leaves and the higher sensitivity of these trees to environmental pollutants, which became visible in the more recent forest diebacks.



## F. Structure and Function of the Root

The root anchors the shoot in the soil. It also serves in the uptake and transport of water and mineral salts as well as the storage of starch and other material. In addition, several syntheses take place, e.g., phytohormones. The organization of the root is different from that of the stem: there is no partition into nodes and internodes. Leaves and stomata are generally absent.

In the longitudinal direction four zones can be recognized (Fig. 34). At the root apex the apical meristem is located. The protection of this most



**Figure 34** Organization of the root. (From Ref. 3.)

sensitive region is provided by the root cap (calyptra). The outer cells of this parenchymalike tissue are continuously shed and replaced by the formation of new cells from the inside. This construction facilitates growth of the root when it penetrates the soil (lubrication function). In addition, the central cells function as statenchyma, which makes possible the orientation of the root in the gravity field of Earth (gravitropism). Sedimenting amyloplasts are used for the perception of gravity. At the vertical orientation of the root they contact the endoplasmic reticulum. This membrane system is found in a dense packing at the bottom of cells. Comparable structures are found in the stem, for instance, in the starch sheaths of internodes of growing stems or in the basal region of leaf sheaths.

The meristematic zone of the root apex adjoins to the elongation zone, which is only a few millimeters long. Therefore only a very small portion of the root is pushed through the soil during growth. In this way distortions or even injuries are prevented.

The root hair zone or differentiation zone follows upward. Here the root epidermis (rhizodermis) forms narrow, tubelike extensions up to 8 mm in length made by local extensions of the cell wall. They may be contorted in their growth between and around soil particles. The thin walls, mainly composed of cellulose and pectic substances, favor the absorption of water and mineral nutrients. There is at most weak cutinization. The construction of the root hair zone leads to a huge enlargement of the surface. Since new root hairs are constantly formed at the anterior end while those at the posterior end are dying, new, actively growing root hairs are constantly getting into contact with new soil particles, when the root advances through the soil. It has been calculated for rye that per day more than 100 million root hairs are formed.

The absorption capacity of the root can be considerably increased by a symbiosis with mycorrhizal fungi. These associations in the root regions are termed *mycorrhizae*. In the field these fungi are more the rule than an exception. This particularly holds true for forest trees, where mycorrhiza formation leads to a suppression of root hairs accompanied by conspicuous morphological changes in the root region. The fungal partner especially improves the uptake of mineral nutrients, particularly phosphate, because of the extended absorption area and the larger surface of the fungal mycelium. There is little doubt that interference with mycorrhiza occurred in previous forest diebacks.

Entirely different is another symbiosis in the root region, the root nodules. In this case soil bacteria of the *Rhizobium* group penetrate through the root hairs of legumes deeply into the cortex, where they cause intensive cell divisions leading to nodule formation. The bacteria in the central area of the nodules fix nitrogen, which is further processed



by the host plant. Eukaryotic organisms are not capable of nitrogen fixation.

Similar structures to the nodules of rhizobia are found in the actinorhiza symbiosis between plant roots of several species, among them alder, *Casuarina* and *Eucalyptus* species, and the nitrogen fixing *Frankia* actinobacteria.

The root hair zone extends upward to the zone of lateral roots. In this region the root epidermis including root hairs is replaced by the exodermis, derived from the outer cortex layer. Because of the suberinization of the exodermis passage, cells with thin walls allow the uptake of water and mineral salts through this region. The lateral roots originate from lateral root primordia formed by the pericycle. The growing lateral roots push their way through the cortical cells and the exodermis.

The anatomical structure of the root differs from that of the stem mainly by a different distribution of the vascular tissue, which is arranged as a radial bundle complex within the central cylinder. Usually the xylem consists of a central solid core of xylem elements with several radiating arms, between which groups of phloem elements are interspersed. The shift of mechanically stable elements to the center of the root indicates a construction of high tensile strength.

The central cylinder is delimited by the pericycle. It flanks the endodermis, the inner layer of the cortex. The endodermis terminates the apoplast space of the root. A bandlike thickening running around the cell on radial and transverse walls, the Casparian strip, serves as a barrier because of the suberinization of the thickened strip. Water and solutes must pass through the protoplast of the endodermal cells to reach the central cylinder, in other words, the symplast passage must be used. For several pollutants in the soil uptake into the root ends at the endodermis.

Roots of gymnosperms and most dicots can perform secondary growth in their older regions. Their initiation completely differs from that of the stem (cf. Fig. 34).

Roots have a dramatic influence on the surrounding soil zone, the rhizosphere. The excretion of  $H^+$  and a variety of organic compounds, the root exudates, leads to surface reactions, which change the chemical and physical properties of soil particles and contribute to the complexation of ions in the soil solution. Root exudates in the form of organic acids also contribute to the solubilization and mobilization of phosphates in the soil. The rhizosphere effect extends to soil organisms whose growth can be influenced positively or negatively. Not only water-soluble (e.g., phenolic) compounds, but also volatile compounds are responsible for this effect. Particularly important is the mucigel, a gelatinous coating of roots that grows in nonsterile soils. Plant slimes, bacterial cells, and their metabolites

as well as organic compounds of the soil contribute to its formation. This improves the contact with soil particles, especially when roots shrink because of high transpiration rates, and provides protective mechanisms, for instance, in acidic mineral soils, against  $\text{Al}^{3+}$ .

Plants not only change their environment, but are influenced by it. The ability to detect these changes and respond by physiological activities is truly remarkable. Important examples are chemical signals, which are transmitted from roots to the shoot during desiccation of the soil, e.g., in the form of abscisic acid, and lead to stomatal closure and growth inhibition (34). On the other hand roots are equipped with sensors to detect humidity. In this way root growth can be directed to regions with available water (hydrotropism). In the presence of rhizotoxic compounds roots react by immediate stopping of growth. This effect can be used for bioassays. An important example is  $\text{Al}^{3+}$  toxicity, especially under acidic conditions. This is of importance in forest diebacks.

## IV. REPRODUCTION

### A. Definitions

The life span of organisms is restricted. Continuity can only be ensured by propagation. In this case, the organisms (parents) produce new organisms (offspring) by generating single- or multicellular germs, which initiate the next generation. In most cases reproduction is also proliferation, if two or more descendants are produced per parent.

Reproduction follows two strategies: (a) maintenance and increase of a population, which is achieved, if available, by vegetative reproduction, but also by sexual reproduction, (b) generation of new combinations of characters within a population, which can only be achieved by sexual reproduction. The important difference is expressed at the cytological level. Sexual reproduction includes the fusion of two cells and their nuclei (karyogamy) followed by meiosis. It is therefore connected with an alternation in the nuclear phase. The considerably more simple asexual reproduction requires neither karyogamy nor meiosis. The formation of vegetative reproductive cells requires only mitotic nuclear divisions.

Mitotic cell division guarantees genetically identical offspring. However, meiotic cell division produces genetically dissimilar offspring because of recombinatory events during this process. The biological relevance of sexual reproduction is the increase of variation within populations, a crucial requirement for the adaptability of organisms to changing environmental conditions as well as evolution itself. An important process is the selection of random mutations in the different combinations of characters. During

the course of time selection can lead to a shift of the frequency of different alleles of certain genes. Therefore a gradual change of populations, which in this way adapt to changes of their environment, takes place. In other words populations increase their fitness. On the other hand, it is likely that environmental pollution impairs fitness.

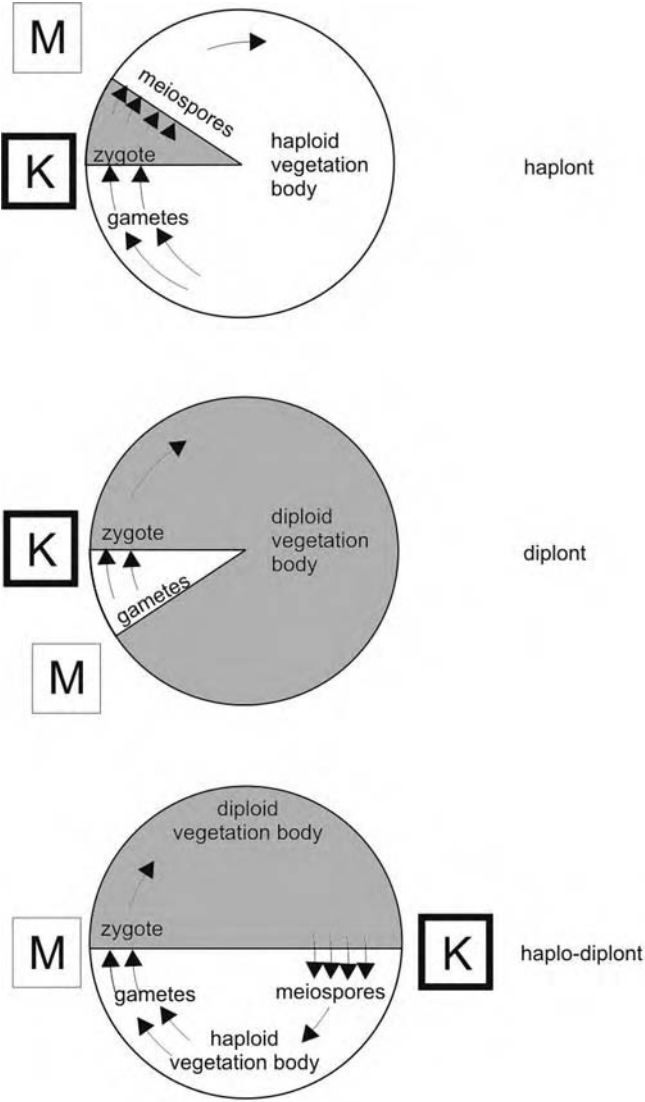
## B. Sexual Reproduction

The diversity of sexual reproduction results from the variety of morphological structures that are available for karyogamy and from the variations concerning the time for karyogamy and meiosis during development. To understand the sequence of developmental cycles, the following definitions are needed: gametes are reproductive cells that fuse and therefore initiate fertilization. The resulting product is the zygote. Gametes can originate in different developmental cycles from meiotic but also from mitotic nuclear divisions. Meiospores always result from meiotic divisions. Further development occurs without karyogamy; usually mitotic cell divisions take place.

## C. Developmental Cycles, Alternation of Generations

The development of each organism starts with a reproductive unit and normally leads to the production of new reproductive units. Such a developmental segment is called a *generation*. Depending on the conjunction of the two cardinal points within the alternation of generations, karyogamy and meiosis, different developmental cycles can be discriminated (Fig. 35):

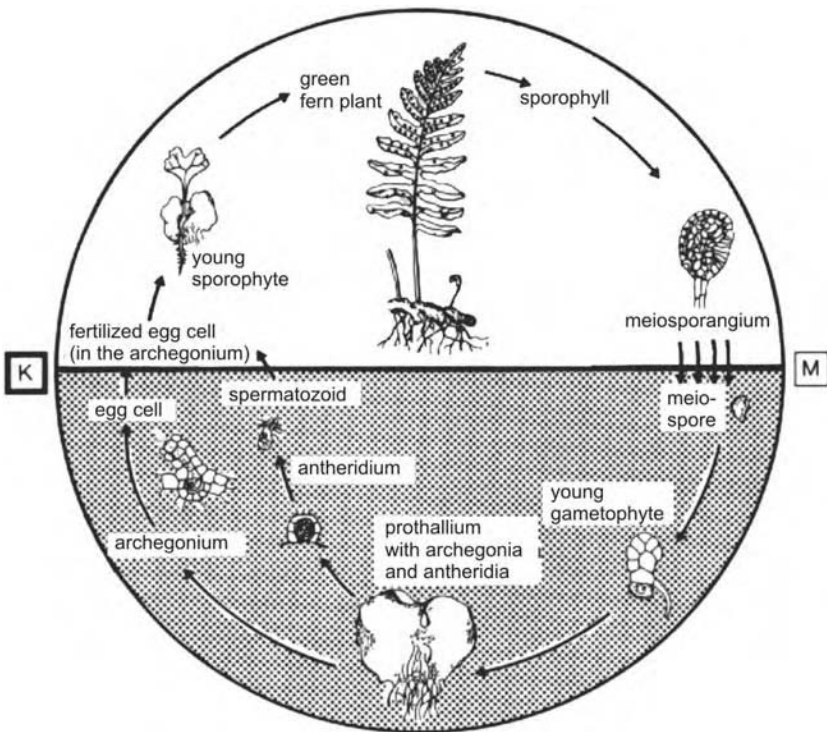
1. In haplonts the diploid phase is limited to the zygote because meiosis directly follows karyogamy. Therefore the vegetation body is haploid. Examples of these developmental cycles are found among algae and lower fungi.
2. In diplonts the haploid phase is restricted to the gametes. The vegetation body is diploid. Meiospores are not produced. In contrast to examples in the animal kingdom there are only a few examples for this mode in plants, which are limited to a few groups of organisms. Diatoms, siphonal green algae, and the oomycetes belong to the diplonts.
3. Haplo-diplonts are most abundant in plants. Separate vegetation bodies occur in the haplo- and the diplophase. Because of the obligatory succession of haplo- and diplophase there is an alternation of two generations, which differ in their reproductive cells. The diploid vegetation body (sporophyte) produces



**Figure 35** Overview of developmental cycles. K, karyogamy; M, meiosis. Diploid generation is marked in gray.

meiospores (emerging from meiosis); the haploid vegetation body (gametophyte) gives rise to gametes (requiring only mitotic cell divisions). The alternation of generations is therefore linked to an alternation of nuclear phase. This developmental cycle is found in many algae and lower fungi. All mosses and higher plants are haplo-diplonts.

This developmental cycle is shown for ferns as an example (cf. Fig. 36). The green plant represents the diploid sporophyte. The leaves bearing sporangia (sporophylls) produce within their sporangia meiospores that serve propagation and dispersal. Each spore develops a haploid gametophyte, the prothallium, where the sexual organs are formed. The male gametes (spermatoocytes) are released from the antheridia. They are chemotactically attracted by the archegonia, which contain a single egg cell each, ready for fertilization. A new sporophyte develops from the zygote. The gametophyte generation is particularly endangered

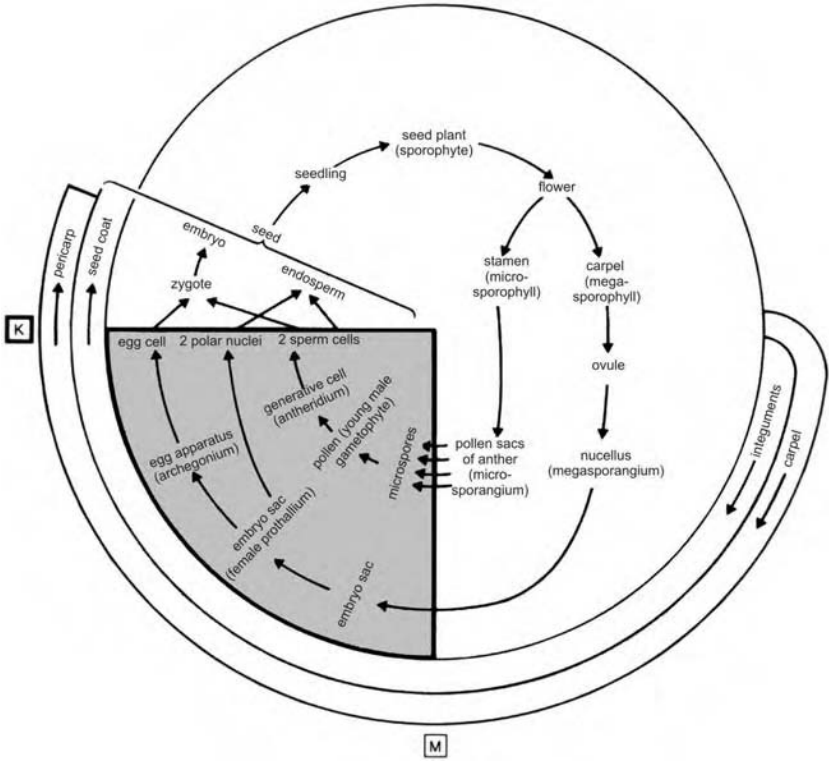


**Figure 36** Developmental cycle of a fern (35, changed).

because the production of gametes and fertilization constitute a most sensitive step. At least the prothallium must be moistened to allow the movement of spermatocytes. Therefore the habitat of the ferns is considerably restricted.

D. Flower: An Overview

Flower formation initiates sexual reproductive cycles in all seed plants. Here the shoot tip converts production from vegetative leaf primordia to flower primordia. In flowers the processes crucial for the sexual cycle, i.e., meiosis and karyogamy, take place. In seed plants similar to ferns sexual reproduction implies the alternation of a diploid sporophyte and a haploid gametophyte generation (Fig. 37). In contrast to that in ferns, the haplophase is extremely short.



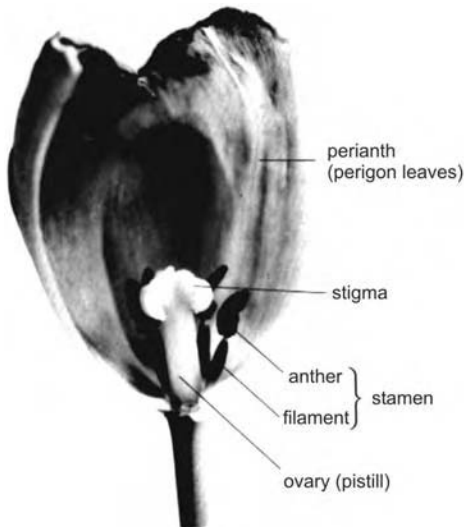
**Figure 37** Developmental cycle of an angiosperm (sporophyte and gametophyte generation; gametophyte generation marked in gray).

The shortening of the gametophyte generation and the placement within structures that first serve the production of spores (namely, in the pollen sacks and the ovules) explain why the terms *androecium* and *gynoecium* (i.e., male and female organs) have been chosen for the entirety of stamen and carpels. Actually they function as sporophylls.

The complicated development of seed plants is the consequence of a perfect adaptation to the life outside the water. Fertilization no longer requires a water film for the movement of the male gametes, as it in the case of fern, but takes place, together with the highly sensitive gametophyte development, within the protective sporophyte tissue. The units of dispersal are no longer the spores but the seeds. These are dormant embryonic sporophytes, equipped with nutrient tissues and surrounded by tissues of the mother sporophyte, the seed coat, for protection.

### E. Structure and Function of the Angiosperm Flower

Regarding morphological characteristics the flower can be considered a reduced shoot with limited growth; its leaf organs have taken reproductive functions. The perianth in dicots is generally divided into petals and sepals, but in monocots it is usually uniform. The stamens function as microsporophylls, the carpels as megasporophylls (Fig. 38). Therefore flowers correspond to an aggregate of sporophylls.



**Figure 38** Structure of an angiosperm flower (tulip).



The carpels of angiosperms form a closed container, the ovary, which usually merges upward, generally into a style, and always terminates at the tip in a stigma, which receives the pollen grains. A carpel contains the ovules (Fig. 39), which later give rise to the seeds. These structures of approximately 1-mm size are attached to the carpel wall with a funiculus. The outer cells of the ovules develop into one or two protective layers, the integuments. At their tip they have a small opening, the micropyle. At this place the pollen tube usually enters, and during seed germination the radicle emerges. The central part of the ovule, the nucellus, serves as a megasporangium. A megaspore mother cell finally gives rise to a haploid megaspore, involving meiosis, and then develops into the mature embryo sac. The most frequently observed stages are the following: formation of an eight-nucleate embryo sac after three mitotic divisions, migration of nuclei, and cell wall formation around nuclei. At the micropylar end of the embryo sac, one egg cell associated with two synergids, the egg apparatus is located. At the opposite pole three antipodal cells are found, whereas in the center two polar nuclei form a binucleate cell, the endosperm mother cell. At this stage the embryo sac corresponds to a seven-celled megaprothallium, ready for fertilization.

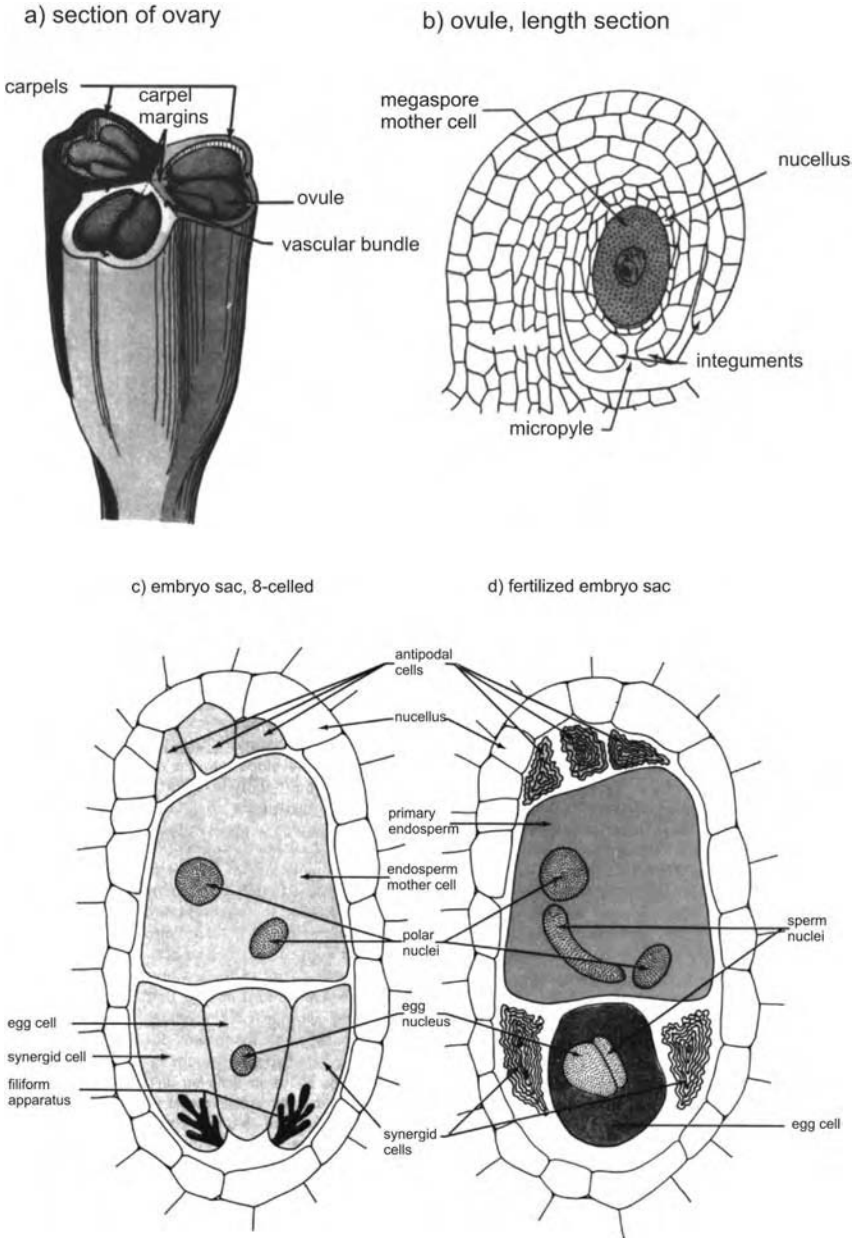
The stamens (Fig. 40) produce four pollen sacs, the microsporangia. Mitotic cell divisions give rise to the haploid pollen cells, the microspores. Already in the pollen sacs further development to pollen grains takes place. These two-celled structures contain a tube cell and a smaller generative cell and correspond to a strongly reduced male prothallium (microprothallium).

The extremely hard wall protects the contents of the pollen grains, the male genome, and is crucial for the transport of the pollen grains to the stigma of neighboring or even more remote flowers.

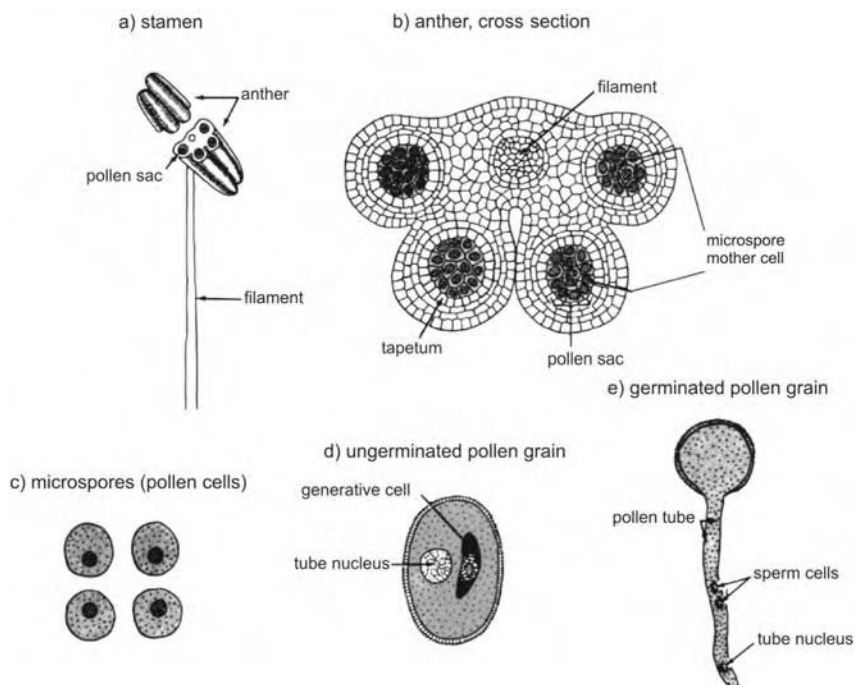
The haploid stages in the pollen sac, but also in the embryo sac, are used in biotechnology for the production of haploid plants (36). They are exploited for the regeneration of haploid sporophytes from gametophytic cells, e.g., microspores. Haploids can be applied in mutation breeding since each mutation, even a recessive one, is already visible in the same generation. In addition haploids can be used for establishing pure lines. The doubling of the chromosomal number, e.g., after colchicine treatment, immediately leads to homozygosity. Therefore haploid plants, which cannot carry out meiosis, serve as transition stages for the production of fertile plants. They have been used successfully for gene transfer experiments.

The dispersal of mature pollen grains takes place in the two-cell or three-cell stage. After pollen reach a compatible stigma (pollination) and water uptake occurs, germination takes place to form a pollen tube. By the end of this stage the generative cell divides into two sperm cells, the male gamete, which now migrates toward the tip of the pollen tube. In many cases





**Figure 39** Structure of the ovary (pistil) and ovules of the tulip. a, section of ovary; b, ovule, length section; c, embryo sac, eight-celled; d, fertilized embryo sac. (From Ref. 26.)



**Figure 40** Structure of the stamen and development of pollen grains. a, stamen; b, anther cross section; c, microspores; d, ungerminated pollen grain; e, germinated pollen grain. (From Ref. 26.)

self-incompatibility genes prevent self-fertilization. In this case germination or pollen tube growth is stopped. The genetic control is either gametophytic or sporophytic. The growth of sperm tubes is among the most sensible steps of plant development. Environmental pollutants heavily interfere with this process.

The pollen tube penetrates the stigma tissue and grows through the pistil until it reaches the ovary. After approaching the ovule, it usually enters the micropyle, penetrates one or more layers of nucellar cells, and then enters the embryo sac. After opening of the pollen tube at its tip fertilization takes place: one sperm cell fuses with the egg cell to form the zygote, the other one with the endosperm mother cell, where the sperm nucleus and the two polar nuclei fuse to form the primary endosperm cell. In consequence, double-fertilization takes place. It requires one pollen tube for each ovule. Whereas the antipodal cells and synergids degenerate, the conditions are set for the further development of seed and fruit. Stigma and style wilt, the petals are shed, and the ovary wall and sometimes additional parts of the

flower develop into the fruit wall, the pericarp. The integuments of the ovules form the seed coat.

Within the ovule the zygote gives rise to the embryo, the primary endosperm cell to the endosperm, which serves as nutritive tissue. Often it is consumed during further seed development. Figure 37 illustrates that three generations are involved in seed structure. The special construction of seed guarantees survival even under extremely unfavorable conditions. In contrast to the function of spore plants such as ferns the seed is the actual unit of dispersal in seed plants, also serving for the preservation of species. Seed formation terminates the reproductive process, starting with flower formation. Fruits are auxiliary structures that have important functions for the protection and dispersal of seeds.

## **F. Vegetative (Asexual) Reproduction**

As the formation of vegetative reproductive units is exclusively based on mitotic cell divisions, daughter organisms are identical to the parent organism. Important examples are twofold and multiple divisions in unicellular organisms and formation of tubers, bulbs, tillers, and plantlets in multicellular organisms. The propagation by cuttings or scions is also an asexual mode of reproduction. In fungi the production of mitospores such as conidia plays major role in the extremely effective propagation.

## **V. DEVELOPMENT**

### **A. Definitions**

The development of a plant comprises all changes that an organism experiences between the stage of the fertilized egg cell and senescence. Formally the developmental process can be divided into several events: growth comprises the irreversible quantitative changes that can be recognized at the cellular level as elongation and cell division growth. In contrast, differentiations include the qualitative changes cells experience during the transition from the embryonic to the adult stage and therefore lead to the diversity of structures and functions. Morphogenesis characterizes those processes that in a multicellular system cause the integration of cells to a specific organization and result in characteristic forms and patterns.

The ordered development is characterized by precise coordination of these three processes. Disturbances lead to pathological events, for instance, formation of tumors if differentiation fails or production of teratoma if morphogenesis is missing.

The course of development is controlled by the genetic information that in each living plant cell is completely retained, even during differentiation. This can be proved by regeneration experiments, in which highly specialized cells dedifferentiate and thereafter develop to a complete organism. Thus development means a sequential expression of different parts of genetic information. Alternatives are possible in plants, which are extremely dependent on their environment. They are called *modifications*. Within the norm created by the genes, extremely different forms can occur under various environmental influences. An important example is photomorphogenesis, development in light, as an alternative to scotomorphogenesis, development in the absence of light.

Environmental factors such as gravity and light play an especially crucial role in the generation of cellular polarity, which is responsible for unequal cell divisions and therefore for the polarity at the level of organs and the entire organism. Where it is required, a continuous change of certain plasmatic properties through the cell or a tissue can trigger different gene activities in different plasmatic areas without changes of the genetic constitution of cell nuclei: the prerequisite for differentiation and morphogenetic processes.

## **B. Hormonal Regulation of the Development**

In plants the coordination among single cells, tissues, and organs is regulated by hormones. In some cases phytohormones have an intermediate position between the glandular hormones, whose production and biological effect are spatially separated, and the phylogenetically older cell hormones of animals, which already act at the site of synthesis.

In the broadest sense phytohormones can be defined as regulators, which are produced in the plant and regulate physiological processes in low concentrations. The classical phytohormones move within the plant from the site of synthesis to the site of action. Hormones trigger only predetermined processes. This mechanism explains their effectiveness in low concentrations. The first step is the binding to a receptor. The competence of the target tissue, i.e., the ability to respond to hormonal signals, can be associated with the presence or absence of respective receptors. The hormone–receptor complex triggers a signal transduction chain that leads ultimately to the primary effects. It is always coupled with signal amplification. Three main mechanisms can be discriminated: (a) change of the permeability of biomembranes, (b) change of gene expression, and (c) direct influence on enzyme activities. These processes are followed by secondary effects, which usually take place at lower speed.

According to their chemical properties seven major classes of phytohormones comprising low molecular weight compounds can be identified. Auxins, cytokinins, gibberellins, and brassinosteroids have mainly positive effects on physiological reactions. Absciscic acid, ethylene, and jasmonates have mainly inhibitory effects, although their multiple effects prevent a strict separation of these two principles. The situation is complicated by the fact that many hormonal effects cannot be traced to a single hormone but to balance among several hormones.

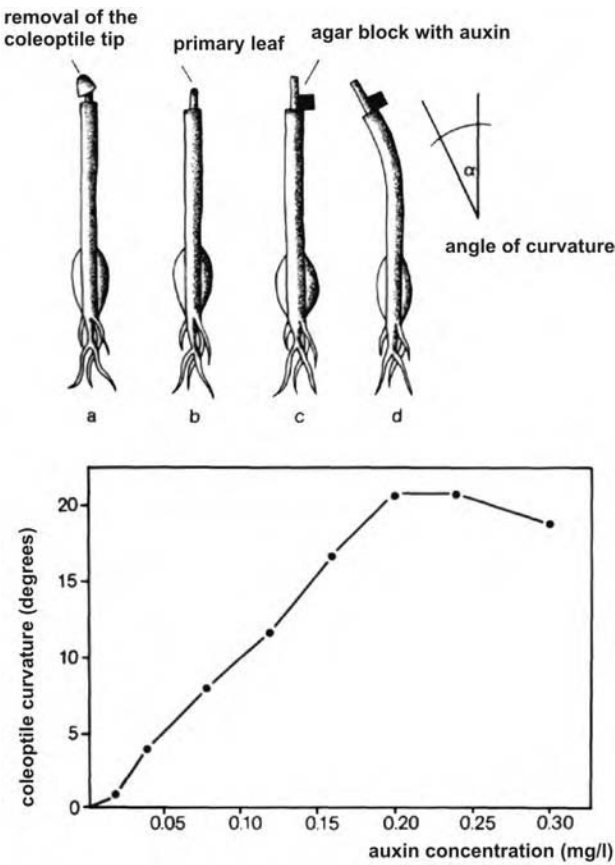
The effective hormone concentration is regulated by its synthesis and degradation, but also by covalent binding to other molecules, for instance, sugars, amino acids, and proteins, as well as their release. Bound forms are either detoxification or storage products, which have no or little hormonal activity.

### C. Auxins

The term *auxin* applies to a group of compounds that stimulate elongation growth of the plant in similar ways. The classical test systems include the bending assay with the decapitated coleoptile stump (Fig. 41), which is based on the strictly polar transport of unilaterally applied auxin. Easier to perform but less sensitive is the segment elongation assay, which is performed with coleoptile or stem segments, for instance, from pea. The auxins include the naturally occurring indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid, and phenyl acetic acid (Fig. 42), as well as many synthetic auxins in which either the indole ring or the acetic acid residue is replaced by other groups. Several herbicides are synthetic auxins and are of specific toxicological interest.

In intact systems the highest auxin concentrations are found in the shoot and root tips. A decrease is observed to the middle. Intensive IAA synthesis takes place in the apical region of the stem including young leaves but also in developing fruits and germinating seeds. The main starting compound for the biosynthesis of IAA is the amino acid tryptophan. The endogenous auxin transport in the intact plant is polar and occurs in the parenchyma, but also in the cambium with a speed of approximately 10–15 mm/h and is energy-dependent. Not entirely clear is the role of a second, nonpolar transport, which can be easily demonstrated after exogenous auxin application to the plant. In this case auxin is transported together with assimilates in the phloem at a speed of c. 10–20 cm/h. Auxin distribution in the plant is not uniform.

Concentrations of free IAA in plant tissues are found at between 1 and 100 µg/kg fresh weight. The actual concentrations result from the equilibrium among synthesis, conjugation, translocation, and oxidative degradation involving IAA oxidase or peroxidase to 3-methyleneoxindole

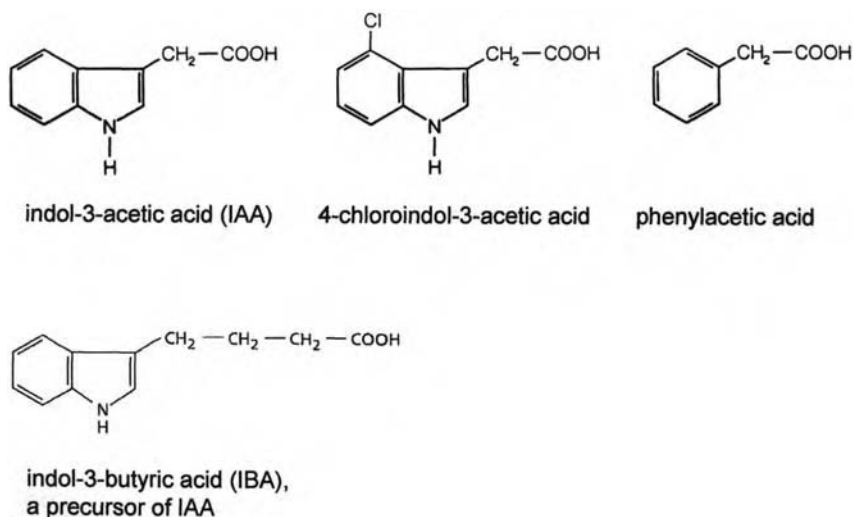


**Figure 41** Bending assay with decapitated *Avena* coleoptiles and quantitative evaluation. (From Refs. 37 and 38.)

and oxindole-3-acetic acid. High auxin concentrations promote ethylene synthesis by stimulating 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. This mechanism explains the similarity of ethylene and auxin effects after application of excessive auxin concentrations.

The auxin effects are manifold and depend on the differentiation of the plant, the available auxin concentrations, and the presence of other phytohormones. Table 3 gives an overview of the most important auxin-regulated processes.

Antiauxins (auxin inhibitors, Fig. 43) can specifically inhibit auxin effects. They include 2,3,5-triiodobenzoic acid (TIBA), which inhibits the parenchymatic auxin transport and causes bushy growth in some



**Figure 42** Naturally occurring auxins.

**Table 3** Multiple Effects of Indole-3-Acetic Acid

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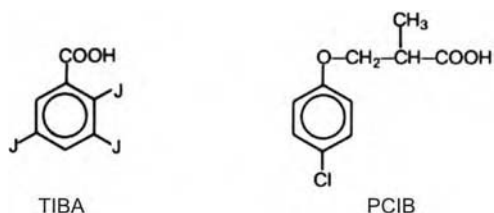
Promotion of cell elongation
Promotion of cell division
Xylem differentiation
Formation of adventitious roots
Apical dominance (apical leaves inhibit sprouting of lateral buds)
Fruit growth
Production of seedless fruits
Determination of sex (formation of female flowers)

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agricultural crops and ornamentals, *p*-chlorophenoxyisobutyric acid (PCIB) with similar effects, as well as cinnamic acid and 9-hydroxyfluoric-9-carboxylic acid.

Polar auxin transport is an important requirement for phototropism and gravitropism but also for the generation of the bilateral symmetry during early embryogenesis.

During recent years several specific auxin binding proteins have been identified in the plasmalemma, tonoplast, ER, and cell nucleus. They include auxin receptors and auxin transporters. The existence of several auxin receptors is consistent with its multiple effects. Different receptors



**Figure 43** Auxin inhibitors. TIBA, 2,3,5-triodobenzoic acid; PCIB, *p*-chloro phenoxyisobutyric acid.

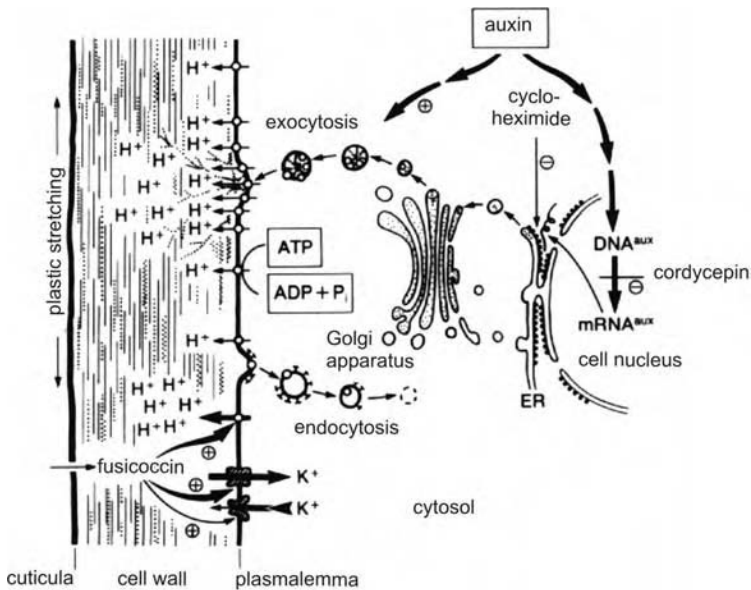
that have been postulated for auxin-induced elongation growth are found in the plasmalemma and the cell nucleus. This view considers the fact that auxin treatment leads to two different effects (39) within a few minutes: (a) acidification of the cell wall followed by an increase of the plastic extensibility of the cell wall, triggering a turgor-driven cell elongation, the acid growth theory directly relates the auxin induced  $H^+$  extrusion by plasmalemma adenosine triphosphatases (ATPases) to cell elongation, (b) specific gene expression and messenger ribonucleic acid (mRNA) and protein synthesis for the production of cell wall components. These events prevent the thinning of the stretching wall. Figure 44 shows a model explaining the primary effect of auxin in relation to cell elongation. It is based on the finding that auxin simultaneously increases the amount of plasmalemma ATPase and membrane flow from the ER to the plasmalemma. The export of exocytotic vesicles provides new  $H^+$ -ATPases, which are incorporated into the plasmalemma. At the same time cell wall precursors, which are required for the wall extension, and enzymes are exported into the apoplast.

The biochemical events during cell extensions are not entirely clear yet. However, there are clues for the existence of expansins. These cell wall proteins trigger cell expansion under acidic conditions (optimum at pH 4.5–5.0) even in vitro (41). One of several auxin binding proteins has been identified as glutathione transferase (42). This cytosolic enzyme, which catalyzes the conjugation of different electrophilic molecules with the tripeptide glutathione, is presumably modulated as a nonsubstrate ligand.

#### D. Cytokinins

The term *cytokinin* indicates promotion of cell division. The first clear proof for the existence of these phytohormones was based on the pith parenchyma of tobacco, whose fully differentiated cells can be stimulated to divide if a cytokinin is applied. All known, naturally occurring cytokinins are derived from an  $N^6$ -substituted adenine. They can be grouped into (a) the  $N^6$

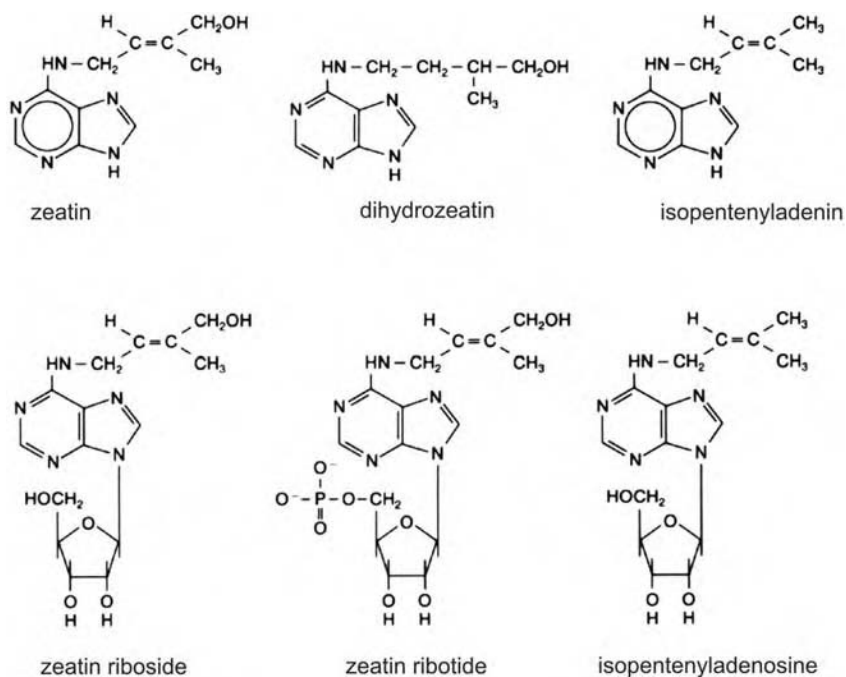




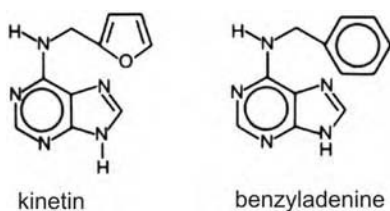
**Figure 44** Model explaining the primary effect of auxin during cell elongation (40). Auxin induces the exocytosis of vesicles and the transcription of auxin-specific messenger ribonucleic acid (mRNA). The effects of the antibiotic fusicoccin (+) and cycloheximide (–) are indicated. DNA<sup>aux</sup>, RNA<sup>aux</sup>: auxin-activated specific nucleic acid sequences. DNA, deoxyribonucleic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

isoprenoid adenine analogs, which are derived from (1) zeatin and its riboside, (2) dihydrozeatin, and (3) from isopentenyladenine, and (b) the N<sup>6</sup> benzyladenine analogs. Figure 45 shows several important cytokinins of the more than 40 naturally occurring cytokinins. In addition to the three groups of bases there are ribosides such as ribosylzeatin and isopentenyladenosine as well as ribotides, e.g., ribosylzeatin-5'-monophosphate. Similarly to the auxins there are also many (at least 100) synthetic cytokinins. Interestingly, the first cytokinin kinetin (6-furfurylaminopurine, Fig. 46) discovered, which was extracted by Skoog and coworkers from several sources including autoclaved herring sperm deoxyribonucleic acid (DNA), belongs in this group. Benzyladenine, which occurs naturally, is of practical relevance, too.

*N,N'*-Diphenylurea (Fig. 47), which occurs in coconut milk, shows cytokininlike activity. It binds to the cytokinin receptor, and its effect is probably structural similarity to the cytokinin riboside ribofuranosyl-purine-6-carbonyl-threonine (Ad-CO-thr). Fungicides of the benzimidazol type (e.g., thiabendazol) show a cytokinin effect.

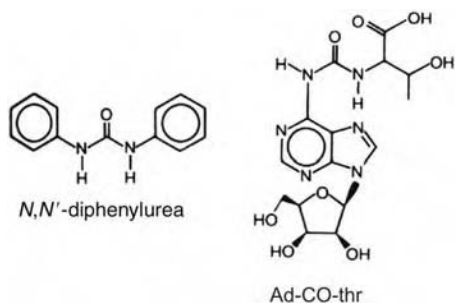


**Figure 45** Naturally occurring cytokinins.



**Figure 46** Synthetic cytokinins.

One of the main sites of cytokinin synthesis is the root tip, from which the transport to other plant parts as a ribotide takes place, especially via xylem. Transport of this phytohormone has also been observed in the phloem. In the leaves there is a transformation to glucosides and free cytokinins. Particularly high concentrations are found in all rapidly dividing tissues, e.g., meristems (including young leaves, young fruits, and tumor tissue), not only higher plants but also mosses, fungi, algae, and even bacteria contain cytokinins.



**Figure 47** *N,N'*-Diphenylurea and ribofuranosyl-purine-6-carbonyl-threonin.

**Table 4** Multiple Effects of Cytokinins

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Promotion of cell division (in the presence of auxins)
Promotion of chloroplast development
Xylem differentiation
Promotion of the development of lateral buds (inhibition of apical dominance)
Regeneration of shoots
Promotion of cell expansion in leaves and cotyledons
Promotion of seed germination
Delay of senescence in leaves and flowers
Inhibition of root growth
Induction of bud formation in mosses

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Synthesis of cytokinins starts with low-molecular-weight precursors: the adenine molecule is derivatized in the N<sup>6</sup> position by a side chain derived from an isoprene derivative. Cytokinins are also found as components of some transfer RNA (tRNA) species, in which they originate by the transfer of an isopentenyl residue to an adenine in the polynucleotide chain. Therefore cytokinins can also be liberated during the degradation of tRNA. This pathway seems to be more important for bacterial cytokinins. The concentration of free cytokinin in plants is in the range of 0.1 mg/kg fresh weight. As is the case for other phytohormones, the cytokinin level results from biosynthesis, degradation by cytokinin oxidase, as well as interconversions with transport and storage forms.

As is the case for auxins, cytokinins trigger a variety of biological effects, which are summarized in Table 4. In horticultural practice the effect of retarded senescence is frequently applied, for instance, to increase

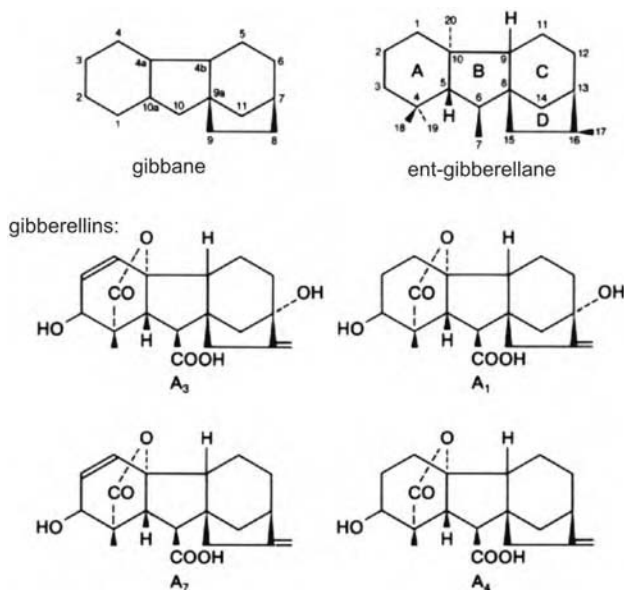
the life of cut flowers or even vegetables. For this purpose synthetic cytokinins are applied. Several phytopathogenic organisms use cytokinins to delay the senescence of certain tissue regions. They are often found as green islands around the source of infection.

Cytokinin receptors have been identified recently. Cytokinin receptor 1 (CRE1) is part of a two-component signaling system. Signal perception by the input domain after cytokinin binding induces a phosphate transfer from a transmitter to a receiver domain as the first step in the cytokinin signal transduction pathway (43). A different effect includes increase of the intracellular  $\text{Ca}^{2+}$  concentration in mosses after binding to a  $\text{Ca}^{2+}$  transport protein, shortening of the S phase in the cell cycle after initiation of DNA replication, binding to a ribosomal protein, and interaction with the redox status of the cell.

### E. Gibberellins

The name of the gibberellin phytohormone group is derived from the ascomycete *Gibberella fujikuroi*, whose imperfect form is known under the name *Fusarium moniliforme*. It had already been discovered before 1900 in Japan that this fungus evokes the symptoms of the *bakanae* disease (the foolish seedling disease). Infected rice plants respond by excessive growth, the plants bending and weakening. Gibberellin, which is secreted by the fungus, was identified much later as the bioactive factor. Later it became known that gibberellins also occur as natural phytohormones, especially in higher plants, but also in algae and bacteria. Today more than 120 forms are known, they are derived from the ent-gibberellane ring system (gibberane skeleton, Fig. 48), a diterpenoid. Only a third is biologically active. For experimental purposes mainly gibberellic acid  $\text{GA}_3$ , which exhibits high activity, is used. The most active forms in higher plants are  $\text{GA}_1$ ,  $\text{GA}_4$ , and  $\text{GA}_7$ .

The main sites of gibberellin biosynthesis in higher plants are the young leaves at the shoot tip, the meristematic tissues of the shoot axes as well as young fruits and premature and germinating seeds. Synthesis starts with pyruvate and glyceraldehyde phosphate, leading to isopentenyl-pyrophosphate and to kaurene as intermediates. These steps take place in the plastids. Kaurene is presumably mobile. It can be moved from the younger tissues to older leaves via phloem. The subsequent steps involve kaurene oxidation, which takes place at the plastid envelope and the ER. The final steps take place in the cytosol and include the conversion of  $\text{GA}_{12}$  aldehyde to  $\text{C}_{19}$  GAs such as  $\text{GA}_1$ . Specific steps can be blocked by growth regulators such as Amo-1618, CCC, or Ancymidol (Fig. 49), which can be used as growth retardants. Practical applications are seen in horticulture as denser leaves and flowers are achieved because of the shorter



**Figure 48** Derivation of gibberellins from the ent-gibberellane ring system.

shoot axes. This characteristic is appreciated by the consumer because of the higher decorative value. In agriculture CCC is used to improve the stability of crops, again because of their shorter stems.

Particularly high gibberellin concentrations, often in the range of milligrams per kilogram fresh weight, are found in immature seeds and fruits. Usually the transport takes place in bound forms, e.g., as GA glucosides or glucosyl esters in the conducting tissues of the plant, but also in a nonpolar form in parenchymatic tissue. In contrast to auxins, exogenously applied gibberellins are easily taken up by the intact plant. The tissues tolerate relatively large concentrations without any damage.

The different biological effects are summarized in Table 5. The GA-induced synthesis of mRNA for  $\alpha$ -amylase and other enzymes in aleurone cells of grains is among the intensively investigated effects of phytohormones. The respective receptors are integrated into the plasmalemma of the aleurone cells. Signal transduction involves the activation of a heterotrimeric G protein followed by a  $\text{Ca}^{2+}$ -dependent and a  $\text{Ca}^{2+}$ -independent pathway. The latter leads to the inactivation of a repressor of GA-responsive genes and therefore to the expression of proteins such as  $\alpha$ -amylase.

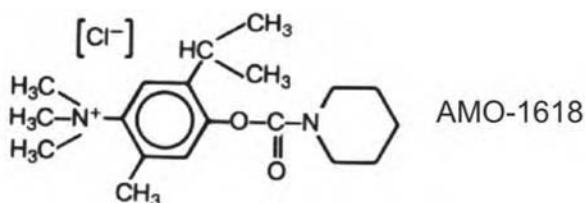
The practical use of gibberellins, which are still obtained from fungal cultures, is particularly seen in horticulture, e.g., for the production of large

**Table 5** Multiple Effects of Gibberellins

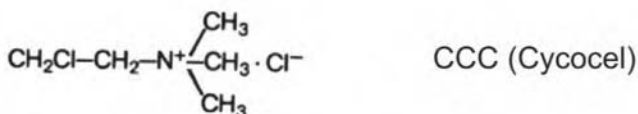
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Promotion of cell extension and cell division
Mobilization of storage material in cereals
De novo synthesis of hydrolases in aleuron cells
Phloem differentiation
Sprouting of dormant buds
Promotion of seed germination
Production of seedless fruits
Promotion of flower and fruit formation
Promotion of cone formation in conifers
Sex determination (formation of male flowers)
Compensation of cold requirement for flowering
Elongation growth in rosette plants

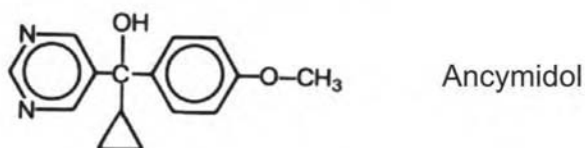
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2'-isopropyl-4'-(trimethylammoniumchloride)-5'-methylphenylpiperidine-1-carboxylate



(2-chloroethyl)-trimethylammoniumchloride



α-cyclopropyl-α-(p-methoxyphenyl)-5-pyrimidine-methylalcohol

**Figure 49** Growth inhibitors that interfere with gibberellin biosynthesis.

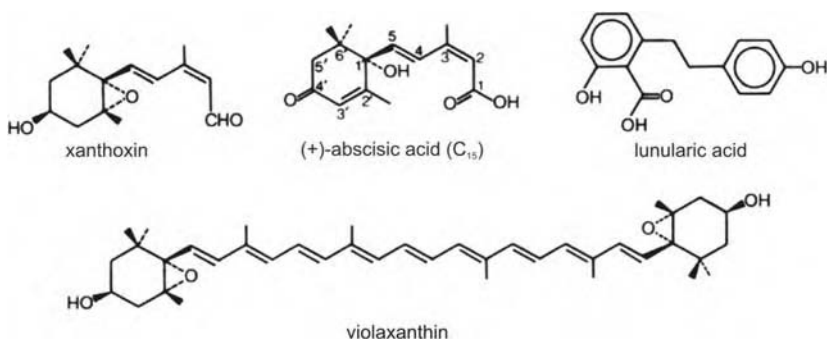
and seedless grapes, the delay of senescence of orange peels, improved flower set in cherries, and improved fruit set in apples and pears.

## F. Absciscic Acid

The plant is adapted to unfavorable climate conditions (e.g., coldness, winter dryness) by a well timed transition into dormancy. For this purpose a series of active processes are initiated, mediated by phytohormones. The consequences are the inhibition and arrest of metabolic and growth processes. Important examples are the formation of winter buds, the fall of leaves, and the initiation of seed dormancy.

The relevance of abscisic acid (ABA), which acts as a stress hormone, has initially been recognized in two different systems. A substance enriched from birch leaves triggered the formation of winter buds after application to buds of growing seedlings. In cotton the dehiscence of young leaves and fruits was triggered by a substance isolated from young seed capsules. It has been agreed to call the bioactive compound *abscisic acid*, although in most systems the phytohormone ethylene has greater relevance for leaf and fruit dehiscence.

Absciscic acid belongs to the class of terpenoids. The sesquiterpenoid ( $C_{15}$ ) compound occurs in the plant as (+)-abscisic acid (Fig. 50). The initial steps of biosynthesis are similar to GA synthesis with isopentenylpyrophosphate as an intermediate, followed by the synthesis of the  $C_{40}$  carotenoid violaxanthin. Whereas these steps are carried out in plastids, the final pathway, the degradation to xanthoxin and finally ABA occurs in the cytosol.



**Figure 50** Absciscic acid and its precursors violaxanthin, xanthoxin, and lunularic acid.

The largest amounts of ABA are found in dormant buds, older leaves, tubers and seeds during dormancy, but also fruits. Stress factors of different kinds generally lead to a fast and dramatic increase of concentration. Loss of turgor is one of the most important triggers. This effect can also be simulated by plasmolysis.

Absciscic acid is transported in both xylem and phloem but is usually more abundant in the phloem sap. Absciscic acid synthesized in the roots can be transported to the shoot through the xylem. The ABA concentrations in the xylem sap of well-watered sunflower plants are between 1.0 and 15.0 nM, whereas the concentrations in water-stressed plants reach up to 3.0 μM.

The oxidative degradation leads to phaseic acid and dihydrophaseic acid, for instance, when the elevated ABA levels after water stress decrease as a consequence of watering. In addition ABA can be inactivated by conjugation to monosaccharides.

Table 6 shows the multiple effects of ABA. A conspicuous effect is the rapid closure of stomata, providing an effective protection against water stress, which again is coupled to an inhibition of nutrient and CO<sub>2</sub> uptake. Water stress causes a redistribution of ABA in the leaf. Under normal conditions (pH 6.3) the undissociated form of ABA is taken up by the mesophyll cells. During water stress, however, the xylem sap becomes more basic and the ABA dissociates. More ABA reaches the guard cells. Receptor binding leads to an immediate inhibition of H<sup>+</sup> export and the opening of several anionic channels, resulting in a decrease of turgor and stomatal closure. It was found in 1984 that the signal transduction chain involves the production of nitric oxide (NO), an intermediate also well known in human physiological processes. There are trends to apply ABA or similar compounds to reduce transpiration in cultivated plants. Several ABA analogs that are available have greater efficiency than ABA (44).

Whereas absciscic acid occurs in higher plants and mosses it is replaced in liverworts and algae by the inhibitor lunularic acid (Fig. 50).

**Table 6** Multiple effects of absciscic acid

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Stomatal closure
Inhibition of cell extension and division
Inhibition of photosynthesis
Initiation of dormancy in buds and seeds
Promotion of leaf and fruit dehiscence (in some systems)
Increase of salt, drought and frost resistance and tolerance

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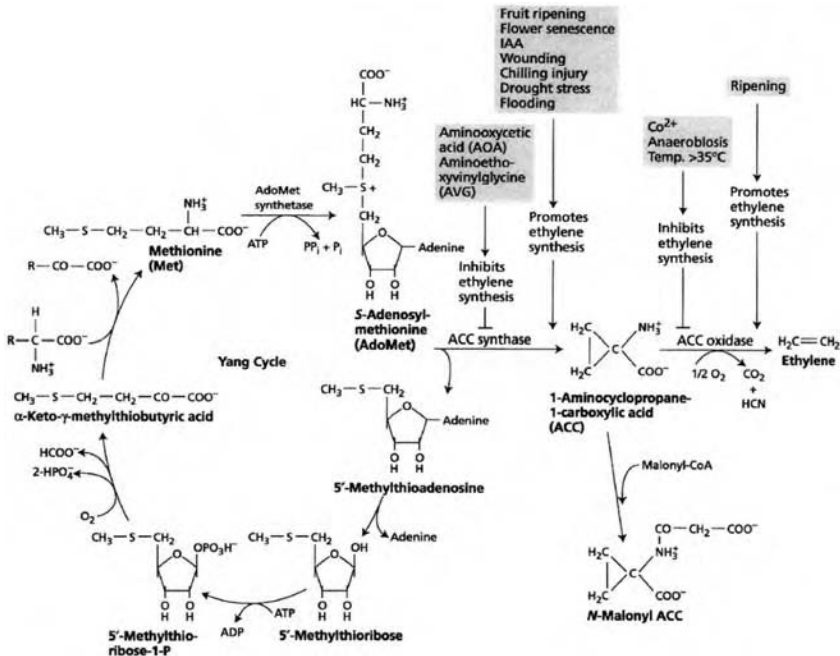


## G. Ethylene

The effects of the gas ethylene ( $\text{H}_2\text{C}=\text{CH}_2$ ) on plants were known long before its relevance as a natural phytohormone was recognized. Its role is described best as that of a ripening and senescence hormone. It is presumably synthesized in all tissues of higher plants, but also in fungi and several bacteria. In addition ethylene may act as a pheromone because it affects not only the producer but also neighbor plants. Effects can already be detected at concentrations below  $1 \mu\text{l/l}$  (ppm). In ripening apples concentrations up to  $2500 \mu\text{l/l}$  are reached.

Ethylene synthesis starts from the amino acid methionine. An important intermediate is 1-aminocyclopropane-1-carboxylic acid (ACC), which is mainly produced in roots and transported within the xylem to the shoot. The main storage site for ACC is the vacuole. Figure 51 shows an overview of the ethylene biosynthetic pathway, including those steps that can be blocked by various inhibitors.

Ethylene synthesis is regulated by the ACC synthase. Stress such as wounding, cold, flooding or drought, but also ethylene itself (autocatalysis!)



**Figure 51** Ethylene biosynthesis. ADP, adenosine diphosphate; ATP, adenosine triphosphate. (From Ref. 45.)

**Table 7** Multiple Effects of Ethylene

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Inhibition of growth (triple response)
Inhibition of chlorophyll synthesis in leaves
Increase of membrane permeability
Asymmetric growth of shoot axis
Hook formation in seedlings
Epinasty of leaf stalks
Promotion of growth in semiaquatic plants (e.g., rice)
Breaking dormancy in buds and seeds
Induction of flowering in Bromeliaceae (pineapple) and Anacardiaceae (mango)
Promotion of abscission in leaves and flowers
Promotion of fruit ripening

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and high IAA concentrations induce the de novo synthesis of ACC synthase followed by intensive ethylene synthesis.

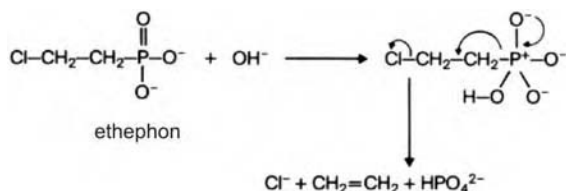
Mechanical influences such as increased pressure, rubbing of plant parts, bending in wind, or wounding, especially by phytopathogenic organisms, stimulate ethylene production. Transport mainly occurs in the apoplast.

Table 7 summarizes the main biological effects. For practical purposes, the role as a fruit ripening hormone in climacteric fruits is especially important. Removal of ethylene with concomitant increase of CO<sub>2</sub> and decrease of O<sub>2</sub> partial pressure (under a controlled atmosphere) can delay fruit ripening for many months.

A similar effect can be achieved by genetic manipulation. Several ripening mutants, e.g., tomato, show delayed fruit ripening and therefore improved storage life, either because ethylene synthesis is blocked or because an ethylene receptor is modified. A particularly interesting example is the introduction of an antisense gene for the ethylene forming enzyme, which catalyzes the conversion of ACC to ethylene. This leads to a dramatic decrease of ethylene synthesis and therefore slower fruit ripening and leaf senescence. These effects can be abolished by ethylene treatment. An unusual response is flower induction, which is employed in plantations of pineapple (Bromeliaceae) and mango (Anacardiaceae) to guarantee uniform flower formation and fruit ripening. For this purpose, ethephon (2-chloro-ethylphosphonic acid) has been applied. It decomposes in the plant under ethylene formation (Fig. 52).

## H. Jasmonates

Jasmonates with the main compounds methyljasmonate (MeJa) and jasmonic acid were originally identified as components of essential oils of



**Figure 52** Ethephon and its decomposition releasing ethylene.

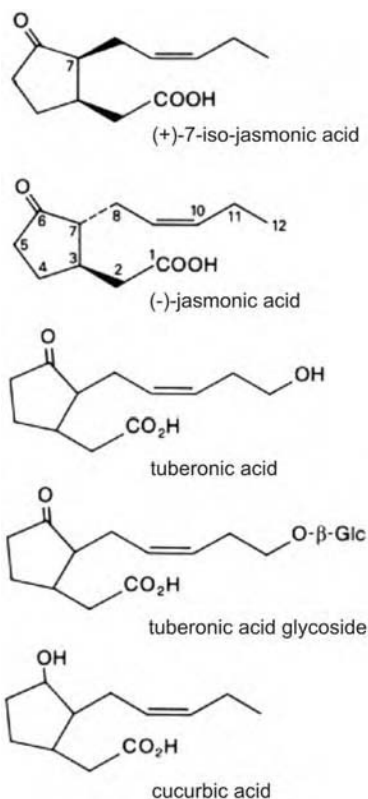
jasmine and rosemary species. Around 1980 Japanese laboratories discovered the inhibitory effects of MeJa on growth of rice seedlings and the promotion of senescence of cut oat leaves. In many ways jasmonate effects resemble those of abscisic acid, especially with respect to stress reactions.

The basic structure of  $\text{C}_{12}$  compounds (Fig. 53) contains a cyclopentanone ring. Because of the two chirality centers in the  $\text{C}_3$  and  $\text{C}_7$  position there are several stereoisomeric compounds. (–)-Jasmonic acid, (+)-7-isojasmonic acid, and their volatile methyl esters are physiologically particularly active. The most efficient one is the MeJa ester, presumably because of its faster uptake. Jasmonate synthesis starts in the chloroplast from  $\alpha$ -linolenic acid (Fig. 54), a 18:3 fatty acid with three double bonds. It is liberated by stress signals as well as jasmonate itself by phospholipase from cell membranes. An oxygenation by lipoxygenase leads to cyclopentanone jasmonates, e.g., 12-oxo-phytodienoic acid (OPDA). The cyclopentenone jasmonates leave the chloroplast either to act as signals or to be further metabolized in the peroxisome, where reduction of the cyclopentenone ring and a shortening of the side chains by  $\beta$ -oxidation take place. The final product is (+)-7-jasmonic acid, which is in equilibrium with (–)-jasmonic acid. The latter can be methylated to yield the volatile methyljasmonic acid (MeJA) (47).

Conspicuous parallels of this pathway to prostaglandin and leukotriene synthesis are noticed. These compounds are released in the animal cell during inflammatory processes and wounding. The hydroxylation of the keto group in the  $\text{C}_6$  position of the ring leads to cucurbit acid (Fig. 53). In addition glucoside and amino acid conjugates can be formed.

Jasmonates occur in most plant organs with concentrations of c. 10 mg/kg fresh weight. Jasmonates are not only found in higher plants, but also in algae and fungi. Table 8 gives an overview of the main responses, among them several inhibitory effects, that usually can be released by cytokinins, the natural antagonists of jasmonates.

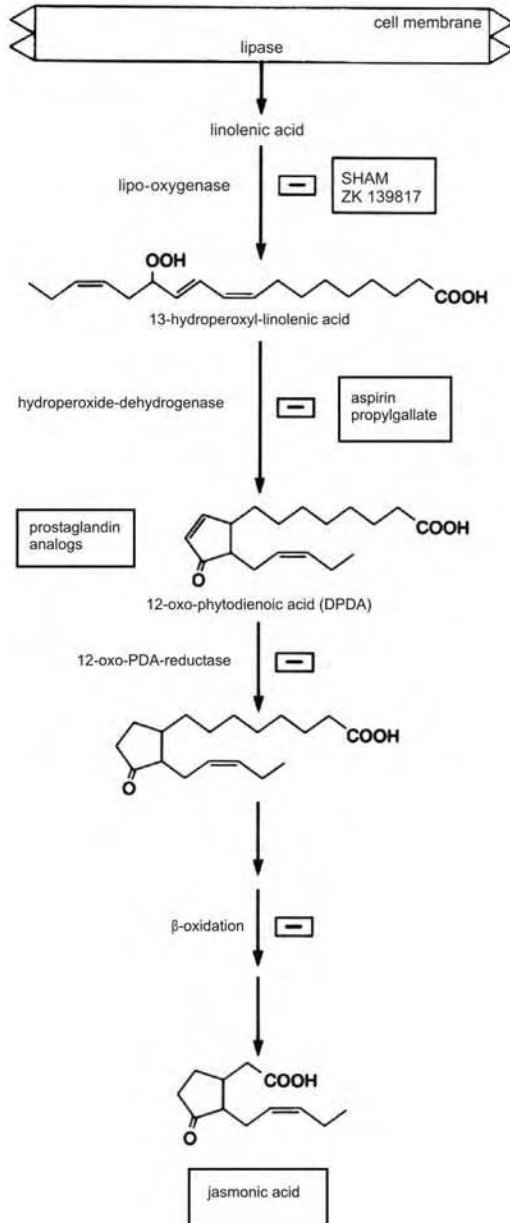
Inhibitions are normally observed in concentration ranges between  $10^{-6}$  and  $10^{-4}$  molar. During seed development jasmonate is synthesized in



**Figure 53** Jasmonate compounds, derived from  $\alpha$ -linolenic acid.

the growing embryo to block premature seed germination; thus jasmonates supplement abscisic acid. An entirely different effect is the induction of tendrillar movement. Weiler (48) has shown that jasmonates play a significant role in signal transduction from touching stimulus to tendrillar movement. This illustrates the relevance of methyljasmonate as a gas because as a systemic signal it affects other plant parts and even other plants.

Several jasmonate-inducible genes have been identified. They include genes for vegetative storage proteins, e.g., in soybean leaves, seed storage proteins and oil body membrane proteins in *Brassica napus* embryos, as well as proteinase inhibitors, which are released during wounding. The most important inhibitor of wound-induced gene activation is aspirine, a salicylate derivative. It also blocks wound-induced jasmonate formation (46). It can be assumed that jasmonates (together with other factors) play



**Figure 54** Biosynthesis of jasmonates and targets of several inhibitors. SHAM, salicylhydroxamic acid; ZK 139817, lipo-oxygenase inhibitor; PDA, phytodienic acid. (From Ref. 46.)

**Table 8** Multiple Effects of Jasmonates

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Inhibition of	embryogenesis, seed germination, growth (including seedlings), callus growth
Promotion of	organ maturation, senescence, chlorophyll degradation
Induction of	tendrillar movement, tuber formation, gene expression of vegetative leaf storage proteins, gene expression of proteinase inhibitors

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a role in the signal transduction chain linking wounding to wound healing, too. In addition to jasmonates there are similar compounds, for instance, cucurbit acid (Fig. 53) from pumpkin seeds, which strongly inhibits growth but on the other hand promotes tuber formation in several plants. Tuberonic acid glycoside is formed in potato leaves during short days. It induces tuber formation, similarly to jasmonates, in the yam *Dioscorea batatas*.

The primary effect of jasmonates includes several targets, among them the  $\text{Ca}^{2+}$  entry into the cytosol; but also the orientation of microtubules and therefore cellulose microfibrils.

**I. Further Phytohormones**

The phytohormone groups mentioned so far do not include all phytohormones and growth regulators.

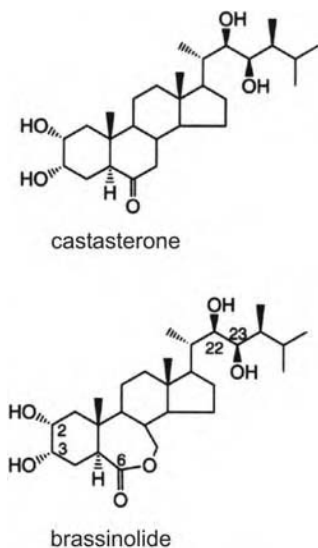
Important regulators of plant development are the polyamines (PAs) a collective name for aliphatic amines. The series of homologous compounds starts with diamines such as putrescine and cadaverine; triamines, e.g., spermidine; and tetramines such as spermine, which also occurs in human sperm. The starting point for these syntheses is putrescine, which is formed in plants by the decarboxylation of arginine, a metabolite of urea.

Polyamines accumulate during stress reactions such as  $\text{K}^{+}$  deficiency, water deficiency, osmotic stress, high concentrations of  $\text{H}^{+}$  or  $\text{NH}_4^{+}$  in the outer medium, and presence of air pollutants, e.g.  $\text{SO}_2$ . Normally PAs occur in high amounts in young organs, and concentrations decrease during

senescence. An exogenous application delays senescence. Practical use is mainly found in protoplast culture. This effect is presumably based on the stabilization of cell membranes. The morphogenetic effects of PAs include the promotion of flower bud formation in tobacco tissue (thin layer cultures), embryoid formation in tissue culture of carrot, and involvement in root formation in leaf explantates (in the absence of callus formation). This effect suggests link to a promotion of cell division.

The important role of brassinosteroids as authentic plant hormones has only been recognized recently. The developmental effects qualitatively resemble those of auxins. Exogenously applied brassinosteroids result in phenotypes that show complex interactions with the classical phytohormones. But they can influence elongation and gene expression independently. They play an important role in the regulation of scotomorphogenesis. Figure 55 shows the structure of brassinolide and castasterone, an intermediate in brassinolide biosynthesis.

Particularly important seems to be the identification of florigen, the flowering hormone, whose identification has been attempted for a long time. Indirect evidence indicates that it is formed in leaves and transported to the shoot apex. Even less is known about vernalin, which is directly produced in the shoot apex as a response to a cold stimulus. It is required in several plants, e.g., winter crops, for flower formation.

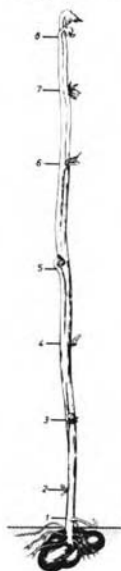


**Figure 55** Structures of castasterone and brassinolide.

## J. Regulation of Development by Environmental Factors

With respect to their development plants as sessile organisms depend much more strongly on their environment than animals. The crucial exogenous factors are light, gravity, temperature, water supply, and mineral salts. Complex interactions are observed between the environmental factors regulating development and the endogenous factors of the organism. In the following the influence of light on plant development is discussed, using photomorphogenesis as an example. Light not only drives photosynthesis but has an additional function as a regulating factor of development. In other words, it serves as an information source during development but as an energy source in photosynthesis. Whereas 8 to 10 light quanta are required to release one molecule of oxygen in photosynthesis, the same amount of quanta per cell is sufficient as a signal for reversing stem growth. A classical photomorphogenic reaction is the inhibition of etiolement by light in the potato plant (Fig. 56). If the plant is grown in dark, it responds by a strong elongation growth of internodes and a minimal growth of leaves (scotomorphogenesis), independently of its pale appearance. In the light rapid growth of leaves and strong inhibition of internodal growth are observed along with greening (photomorphogenesis). This developmental

Control plant grown in darkness



Plant grown in light



**Figure 56** Influence of light on the growth of a potato plant. (From Ref. 49.)



strategy guarantees that the plant only invests its limited reserve of nutrients in internodal growth until it reaches light. Then reversal of growth favors photosynthesis and finally guarantees independence from storage material.

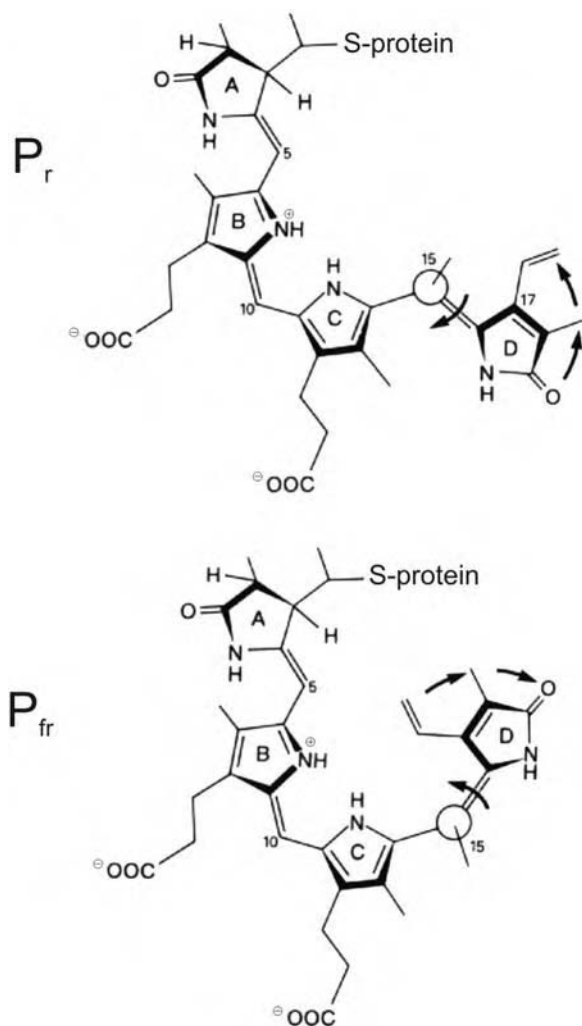
This example clearly shows that light exerts quantitative control in photomorphogenesis on the speed of development. The existing pattern, which is given here by the number of nodes and leaves (cf. Fig. 56), does not change. Photomorphogenesis as well as photoperiodism, the regulation of flower formation, dormancy, and further developmental steps, are influenced by day length. In this case the phytochrome system, which is a cytosolic chromoprotein with a molecular weight of 120 to 130 kd, serves as the photoreceptor. Its chromophoric group is an open-chained tetrapyrrol covalently linked via its A ring to the apoprotein (Fig. 57).

This pigment system converts the energy of absorbed light into a structural change. One of the prominent features of this light sensor is the fact that it exists in two different forms with different spectral sensitivities. These forms can be converted into each other after appropriate irradiation.

In the dark the inactive bluish form is synthesized. Because of its absorption maximum in the red it is referred to as  $P_r$  (Fig. 58). After irradiation with red light  $P_r$  converts into the physiologically active greenish form, which absorbs far red light ( $P_{fr}$ ). Irradiation with far red light restores the inactive initial form  $P_r$ .

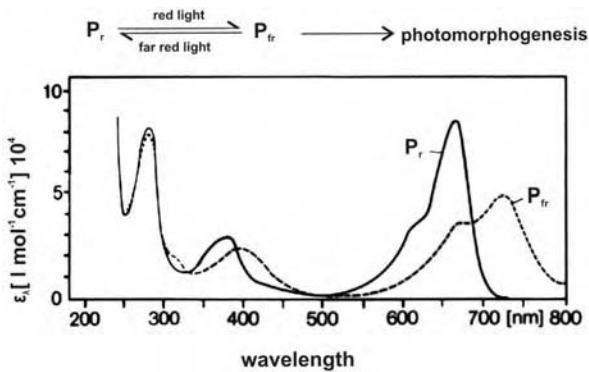
The activation of phytochrome leads to a chemical change of the chromophore. The first step is a Z/E isomerization (cf. Fig. 57), followed by a conformational change in the N-terminal region of the apoprotein. This leads to a cascade of individual reactions, members of signal transduction chains. They lead to different individual reactions such as gene expression (for instance, the transcription of the RUBISCO and *cab* gene), chloroplast rotation, or opening of ion channels. It seems to be clear now that upon photoconversion to the  $P_{fr}$  form, phytochrome migrates into the nucleus, where it activates the expression of primary target genes, many of which are transcriptional regulators (52).

The physiological and ecological relevance of phytochrome is not restricted to the absorption of available light. It also can detect changes of the spectral composition of light by the  $P_{fr}/P_r$  ratio and respond appropriately by changes of growth and development. The spectral composition of light changes in different environments, particularly in the red part of the visible spectrum. For instance dense foliage filters out considerable amounts of the red but not of the far red spectral region. Scattered light and shade from neighboring plants are also enriched with far red. The transition of phytochrome into the inactive form triggers promotion of growth, an optimal strategy to guide the plant to more favorable photosynthetic conditions. In this way light-dependent seeds are



**Figure 57** Chromophore of phytochrome  $P_r$  and  $P_{fr}$ . (From Ref. 50.)

prevented from germinating under unfavorable conditions in upper soil layers that still can be reached by light, since the proportion of red light decreases more strongly with increasing depth than the proportion of far red light. This property is important for light germinating seeds in suboptimal positions. On the other hand, phytochrome enables the seedlings to follow the optimal strategy of growth.



**Figure 58** Absorption spectrum of phytochrome  $P_r$  and  $P_{fr}$  from oat seedlings. (From Ref. 51.)

The spectral composition of light changes during the day. The ratio of r/fr intensities is larger at midday than in the morning and evening. By means of phytochrome the plant receives information on the time between sunrise and sunset and can adjust its growth intensity accordingly, i.e., promotion during night, repression during day. The plant also receives information on day length, which together with temperature sensing can be translated into informations about the season. This remarkable achievement is based on the existence of an endogenous clock, which generates oscillations in a 24-h rhythm. The coincidence of the light phase with defined phases of the endogenous clock allows very exact measurements of time. Usually appropriate light programs, i.e., short days or long days, have to be repeated several times in order to trigger developmental changes such as flower formation, entry into dormancy, leaf abscission, sex determination in flowers, and tuber formation. These qualitative changes, which exert a long-term control on the entire developmental pattern, are photoperiodic reactions. A photoperiodic stimulus, in which a cyclic light program, such as the natural change between light and dark, is presented, is necessary. Table 9 gives an overview of important phytochrome-controlled reactions.

It is now known that phytochrome is encoded by a multichain family. Each phytochrome-mediated response is regulated by a specific phytochrome or by an interaction between specific phytochromes. The different forms exhibit differences in their protein part. Type I phytochrome plays a major role in etiolated seedlings. Its active form is extremely labile. It regulates growth of seedlings and their ability for greening. In *Arabidopsis* species, it corresponds to phytochrome A. Type II phytochrome has major functions in the green plant. It occurs in several forms, termed *phytochrome B–E* in *Arabidopsis* spp. The generation of several mutants that etiolate in

**Table 9** Phytochrome-regulated reactions using angiosperms as an example

<b>1. Photomorphosis</b>	<b>2. Photomodulations</b>
Seed germination	Leaf movements
Inhibition of stem growth	Chloroplast migration and rotation
Opening of the hypocotyl hook	Protoplast swelling
Growth of leaves (cotyledons, adult leaves)	Regulation of membrane potentials and ion fluxes
Promotion of root formation	
Phototropic and gravitropic reactions	<b>3. Photoperiodism</b>
Degradation and translocation of storage material	Flower formation
Synthesis of flavonoids, carotinoids, and ascorbate	Dormancy of buds
Synthesis of phytohormones	Tuber formation
Change of gene expression	Determination of sex

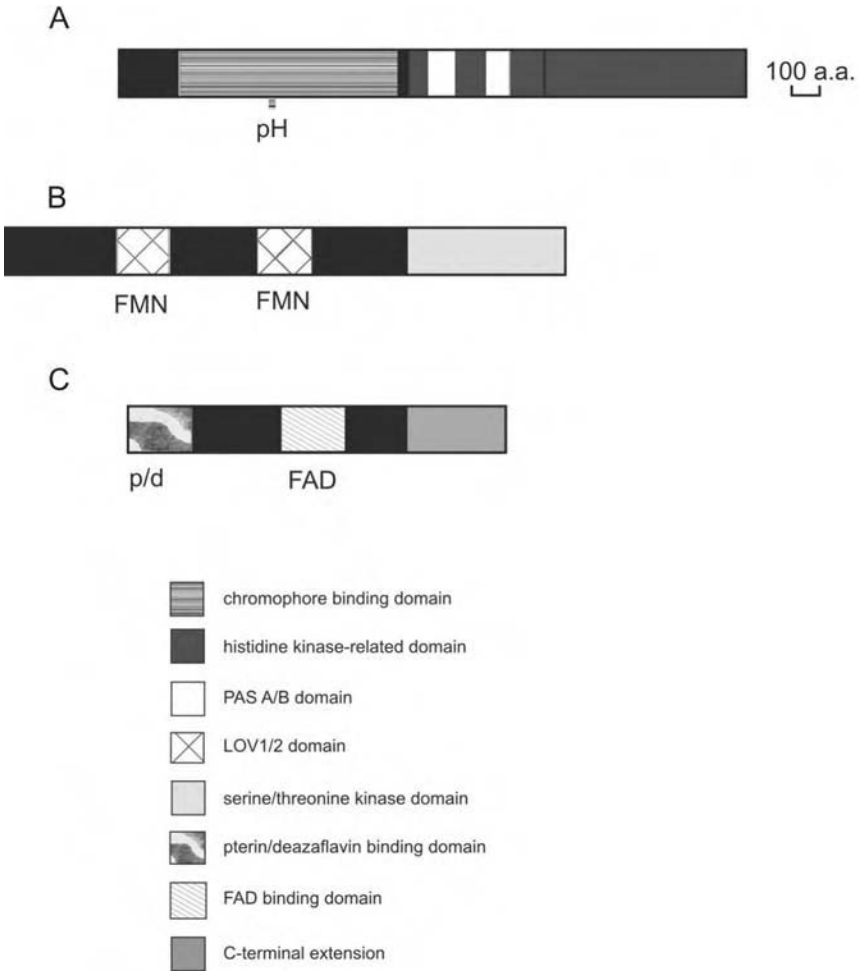
the light or show photomorphogenesis in the dark has revealed many details about the role of individual phytochromes. Figure 59a shows a scheme of the phytochrome holoprotein with the different functional domains. It is now clear that phytochromes are not only light sensor proteins but also light-regulated protein kinases. The  $P_{fr}$  form has auto- and transphosphorylation activity, interacting with signaling intermediates (54). In this way phytochrome is part of a two-component system with striking similarities to their bacterial counterparts.

## K. Blue-Light Receptors

Plants do not rely on a single group of photoreceptors. In addition to phytochromes absorbing in the red light region, blue-light receptors utilize the blue-light region of the spectrum. Phototropins are responsible for the regulation of phototropism, chloroplast migration, and stomatal opening (55). A model for the domain organization is shown in Fig. 59b.

Cryptochromes (Fig. 59c) are a second class of blue-light receptors. They regulate dietiolation, entrainment of the circadian clock, and floral initiation. In contrast to phototropins, which use flavin mononucleotides (FMNs) as chromophores, cryptochromes employ flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate as chromophores.

Finally the carotenoid zeaxanthin is believed to be the blue-light sensor in guard cells. In this case the signal transduction chain is initiated by the excitation of zeaxanthin by blue light. Then the signal is transmitted to the cytoplasm, where it activates a serine/threonine kinase, which phosphorylates and thereby activates the  $H^+$ -ATPase (56).



**Figure 59** Domain organization of plant photoreceptors Ref. (53): A, phytochrome; B, phototropin; C, cryptochrome I. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; p/d, pteridin/dazaflavin binding domain; PAS A/B, Per-Arnt-Sim-domains; LOV 1/2, light, oxygen or voltage domains; a.a., amino acid residues; pH, phytochrome chromophore.

Little is known about the ultraviolet B (UV-B) receptor. It provides information on the presence of damaging UV radiation in the region between 280 and 320 nm. The plant reacts with a series of protective responses, for instance, the synthesis of UV-absorbing pigments such as

flavonoids, particularly in the upper epidermis, reflecting wax layers and an increased leaf diameter with decreased leaf area.

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# 2

## Plant Stress: Avoidance, Adaptation, Defense

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### I. INTRODUCTION

About 3.5 billion years ago, the first light utilizing organisms had only one “cyclic” photosystem. Thus, for the purpose of carbon fixation they had to use exogenous reductants such as hydrogen sulfide or hydroxylamine. The first “energy crisis” arose when these reduced compounds were exhausted (oxidized) in aqueous environments. The solution of the problem for the ancestors of cyanobacteria was the “invention” of photosystem II, i.e., a second photosystem containing a modified chlorophyll with an  $E'_0$  as high as +830 mV, thus allowing them to utilize water as an inexhaustible electron source at zero cost. This novel photosystem produced oxygen, protons, and electrons in a light-dependent reaction involving manganese as catalytic redox converter as an electron trap with water as donor. This strategy was so efficient that it allowed the assembly of high densities of organisms, accumulating as pure carbon, geologically designated as graphite (coal is approximately 2.5–3 billion years younger!).

From this time other unicellular organisms devoid of chlorophyll (heterotrophs) took advantage of these novel “unlimited energy” cells, using them as a food source or even incorporating them as cellular organelles in the sense of photovoltaic elements: coevolution started and multicellular organisms could develop.

However, the trade-off of water splitting by photosystem II was oxygen toxicity. In the first few of hundred million years the problem was not so dramatic since most oxygen was bound and sedimented by the process of iron (II)

oxidation. An intermediate period might have allowed rereduction of oxidized nitrogen (nitrate respiration), thus supporting the original one-photosystem organisms as well as primitive heterotrophs living without oxygen. Later, especially when multicellular (land) plants arose and oxygen accumulated in the atmosphere, they replaced nitrate respiration with the more efficient oxygen respiration utilizing the “counterpart” of water splitting, namely, water formation via oxygen reduction by cytochrome *a/a3*. This system involved both iron and copper as analogous redox converter, now functioning as oxygen trap.

In both cases, water splitting and water formation, oxygen formation and oxygen reduction to water are four-electron steps. Thus, another problem arose: four electrons could not be transferred simultaneously, but step by step, thus involving superoxide, hydrogen peroxide, and the OH radical as intermediates. Since these intermediates are of high chemical reactivity, the mechanism of their production had to be cryptic with excellent insulation to its surroundings, similar to an atomic power plant. Other redox systems in endogenous metabolism present in almost all cellular compartments are also thermodynamically able to reduce oxygen, potentially producing the species mentioned (discussed later). All aerobic organisms therefore had to synthesize antioxidants and develop antioxidative strategies in order to survive. In the following, phototrophic (algae, higher plants) and heterotrophic aerobic multicellular organism (bacteria, fungi, animals) developed cooperative and adaptive strategies of detoxification where-by the heterotrophs again took advantage of the much better synthesizing capacities of the plants: they just “forgot” to build bioenergetically expensive (adenosine triphosphate [ATP] consumption) molecules such as aromats—with some exceptions. Reactive oxygen species—(ROS)—detoxifying systems were developed synergistically and allowed both plants and animals to utilize oxygen activation as defense systems (respiratory burst) exhibiting homologous external and internal battlefields such as the apoplasts of the plant and the phagosome of the animal. Traditional and modern medicine use microbial and higher plants’ products, i.e., their antioxidants (1–3) as drugs and in preventive therapies. Since so many similarities and analogies have been observed in animals and plants in the last decade, human, animal, and plant pathological reactions must be compared in order to understand the underlying common mechanisms and mutual benefits (4).

## **II. OXYGEN STRESS IN PLANTS**

### **A. Induction of Plant Stress**

The term stress stems from physics (applied mechanics) and has been extended and uncritically introduced in medicine and botany. In general

mechanics stress is clearly defined as the point or degree of bending of an elastic system at the very point of just symptomless reversibility and irreversible deformation or break.

In medicine and botany, stress is supposed to indicate all situations beyond normal, in which the individual or organ is supposed to suffer, as defined by the observer, i.e., the human.

Since all organs of higher plants (with some exceptions) perform aerobic metabolism and are thus subject to activated oxygen species, oxygen “oversaturation” and thus possibly oxygen “stress” may arise under various different conditions.

Defense reactions against pathogens in both plants and animals produce reactive oxygen species (ROS) in several cellular compartments by different mechanisms. Thus for protection against damage antioxidative strategies have been developed.

In this chapter basic reactions operating during oxidative stress situations are discussed, and certain pro-oxidative situations and antioxidative processes in plants and, to a certain extent, in animals are considered.

## 1. External Impacts: Air Pollution and Radiation

Ozone and SO<sub>2</sub> as air pollutants and the increase of ultraviolet B (UV-B) as an effect of the decrease of the stratospheric ozone layer and radioactive deposition (Tchernobyl remainder) must be seen as potentially toxic to plants.

Radioactive deposition is no longer an immediate toxicological threat to plants (injury) and can only be seen as a potential impact to consumers (animals and humans: damage in the sense of monetary losses).

Ultraviolet damage in plants is not much of a problem because of the plants' capacity to establish protective systems on the basis of a complex set of UV-absorbent phenolics (5).

SO<sub>2</sub> in most regions of the industrial world (Western Hemisphere) is also no longer a serious problem because of the binding with CaO (forming gypsum) after the burning process in the power plants; in former times, when SO<sub>2</sub> concentrations in industrial regions or in their downstream exhaust could reach up to 1000 µg/m<sup>3</sup> they caused serious pathological problems in both animals and plants (6; see also Chapter 4).

Ozone, however, may reach concentrations in the atmosphere that cause plants to react and seems to be of higher relevance to plants than to animals and human. After ozone treatment of wheat leaves a decrease of the photochemical capacity was observed along with a stable electron paramagnetic resonance (EPR) signal (that appeared to be time, and

concentration-dependent) that had similarities to the ubisemiquinone radical. This EPR signal seemed to be associated with permanent leaf damage (7). Ozone, on the other hand, has been shown to act as an abiotic elicitor, inducing oxidative defense and has been used as a tool for analyzing stress responses in terms of predisposition to pathogen attack (8,9; see Chapter 10). Some plants have developed protective devices (scavenger systems) that react in the gaseous phase, rendering the plant more or less resistant: Isoprene emission (which is observed in many plants under atmospheric conditions favoring tropospheric ozone formation) is an example of the synthesis of a volatile antioxidant molecule that protects against ozone damage (10). Natural volatile terpenes, on the other hand, act as important signals and represent the basis of numerous herbal drugs used in the treatment of pain, cold, bronchitis, and gastrointestinal diseases (11). Thus, abiotic stressors/effectors may lead to signals within the plant opening metabolic pathways and yielding cross-resistance, for example, tolerance/resistance against pathogens such as viruses, bacteria, or fungi in the plant, where calcium and ROS have been identified as key messengers of the abiotic signal chain (12).

## 2. Internal Metabolic Events in Green Plants Forming Reactive Oxygen Species

Stressors (cold, drought, and many others), cause internal oxygen activation via feed back of metabolic blocks to the photosystems (see earlier discussion). External stress always has a change of internal metabolism as a consequence, mediated by a complex interaction of hormones that are synthesized *de novo* or released from internal stores. Adaptation to drought, for example, is counteracted by stomatal closure. This event is initiated by the hormone abscisic acid and mediated by  $H_2O_2$  produced via a reduced oxidized nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (13).

Green plants have adapted to extremely different environmental conditions. Since plants, in contrast to animals, cannot escape, they have to either adapt to the different stresses (Fig. 1) or to die. Depending on the strength of these abiotic or biotic impacts, several symptoms indicating the deviation from normal metabolic conditions may be expressed.

Most of these visible or measurable symptoms have been shown to be linked with oxygen activation (13–16), in which a transition from heterolytic (two-electron transitions) to increased homolytic (one-electron transitions) reactions is principally observed. Homolytic reactions create free radicals. We thus address these situations as oxidative stress.

These stress situations are generally counteracted by a parallel increase of radical scavenging processes or by preexisting or *de novo* synthesized

The Seven “Stress” – Factors

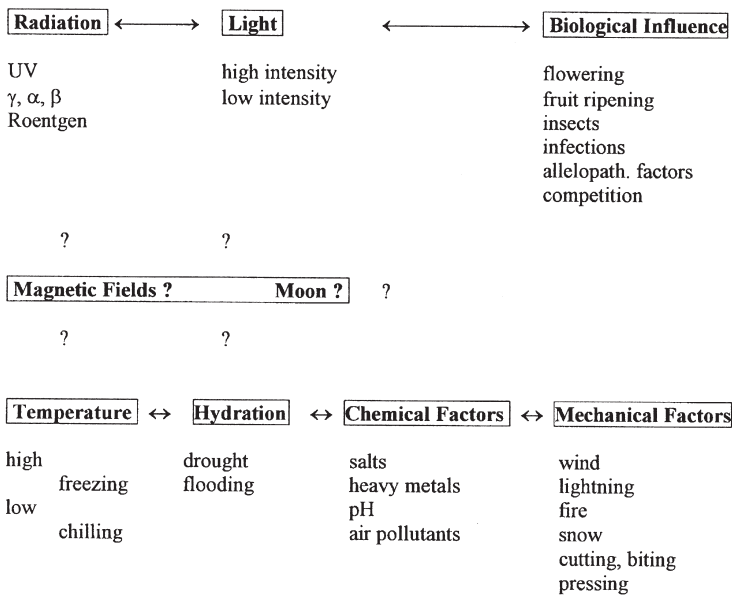


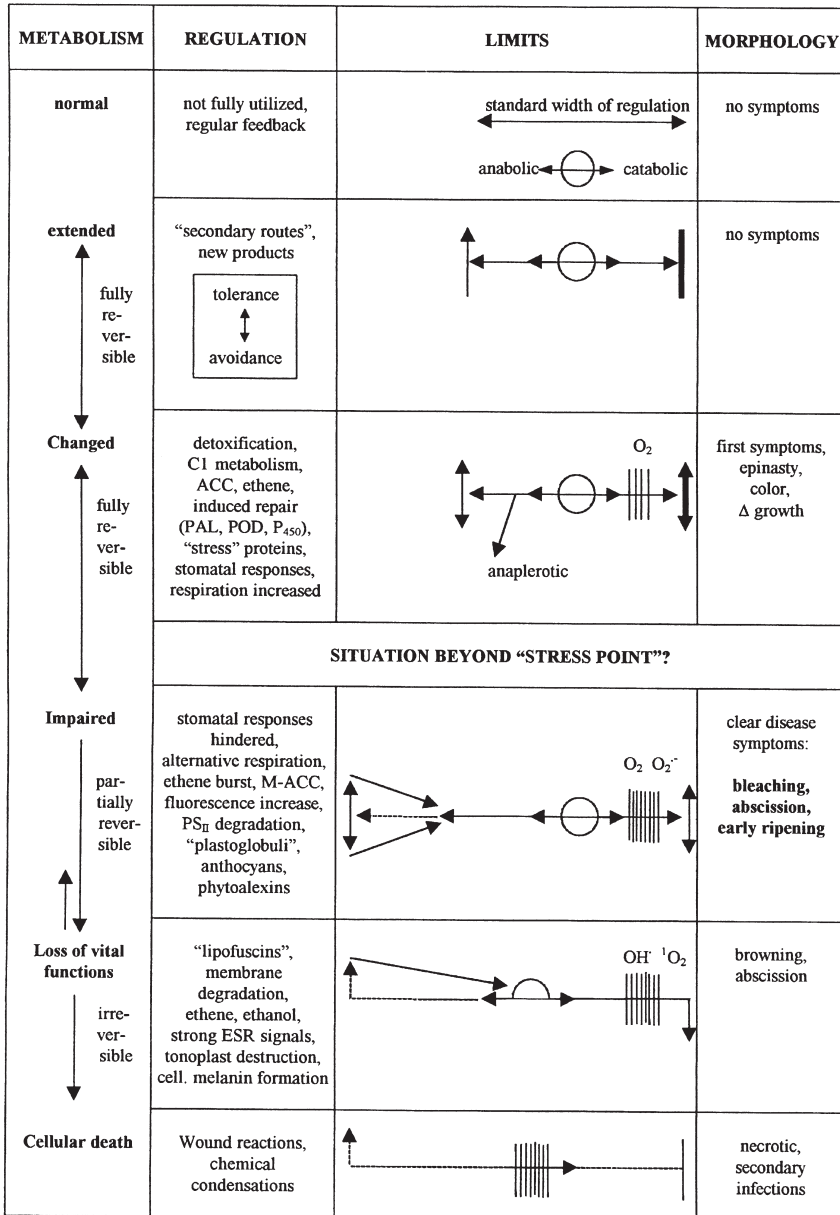
Figure 1 Biotic and abiotic stress factors. (Adapted from Schlee 1992)

compounds that function in detoxification of deleterious ROS and thus metabolic control within certain limits. At advanced or prolonged stress conditions this control may be gradually lost and chaotic radical processes may dominate. Finally, lytic processes induce cellular and tissue decompartmentalizations yielding visible necrosis. This stress cascade leading to progressive symptoms of disease, together with adaptative and regulative counteraction in the “first part” (before a defined stress point?), is shown in Fig. 2.

3. Stress—Avoidance and Defense Strategies of Green Plants

Every episode during this cascade is characterized by the balance between pro- and antioxidative capacities. Thus, in oxygenic photosynthesis, redox regulation by the electron transport system seems to govern growth and development of green plants by a broad range of signals:

1. Redox signals from integral PSII components such as “bound” plastoquinones Q<sub>A</sub> and Q<sub>B</sub>, and cytochrome b-559.



**Figure 2** The stress cascade in plants. ACC, 1-aminocyclopropane-1-carboxylic acid; PAL, Phenylalanine-ammonia-lyase; POD, Peroxidase; P<sub>450</sub>, Cytochrome P<sub>450</sub>; n-ACC, nalovinyl-ACC; PSII, Photosystem II.

2. From the interchain components, mobile plastoquinone and cytochrome complex b/f.
3. Redox signals from photosystem I (PSI) factors and products such as ferredoxin, thioredoxin, hydrogen peroxide and superoxide, NADPH, and soluble redox factor such as reduced glutathione GSH (17).

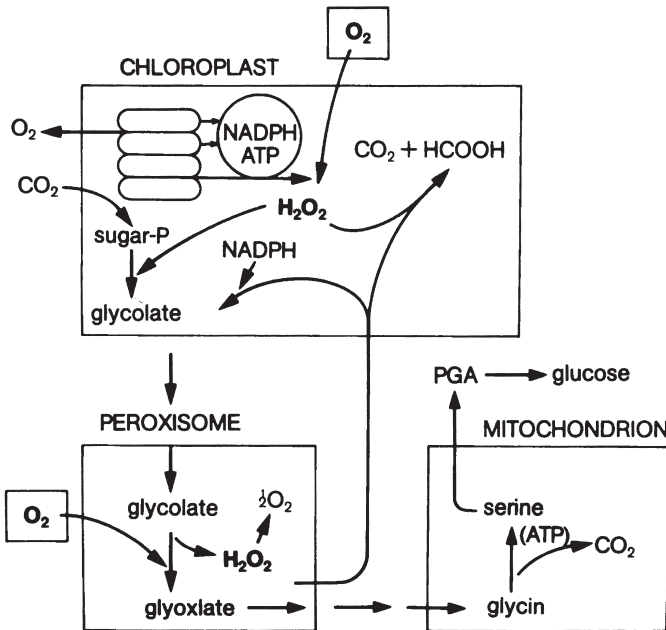
All these signals, together with redox pairs that maintain “valve functions” such as malate/oxalacetate and operate as a redox shuttle, are thought to couple the redox system of the chloroplasts with the nucleus, thus inducing protein (enzymic) responses (18).

In the beginning the photosynthetic system was designed to minimize electron pressure arising from photosystem II (PSII) on an organizational level by the following processes:

1. Enhancing cyclic electron flow across photosystem I; this occurs via reducing the optical diameter, i.e., the light harvesting systems of PSII (LHCII) by transfer and integration of the chlorophyll–protein complex into photosystem I (19) via phosphorylation. Molecular recognition dominates the organization of the thylakoid structure and function (20), and nonphotochemical quenching of excited states supports energy dissipation, thus averting photodamage (21).
2. Cyclic electron flow across photosystem II, induced by a strong pH gradient in cooperation with the water–water cycle (discussed later), thus reducing the electron pressure by PSII (22).
3. The process of photorespiration, connecting several cellular compartments (Fig. 3) by “carbon idling” and enhancement of C-1 metabolism (23–25), producing peroxide, which is detoxified by several mechanisms.
4.  $\text{H}_2\text{O}_2$  cycling (water–water cycle) via the Beck–Halliwell–Asada cycle (26,27) as outlined in Fig. 4.
5. The xanthophyll cycle (28) protecting PSII.
6. Photoinhibition by inactivating the electron “outlet” of PSII via radical-induced degradation of the D-protein.
7. Via the novel plastid terminal oxidase (quinone–oxygen oxidoreductase) resembling the cyanide-resistant alternative oxidase linked to carotene desaturation that is responsible for chlororespiration (29). The LHCII phosphorylation (a), photoinhibition (f), and chlororespiration (g) are tuned by the redox state of the plastoquinone cycle, which seems to play the pivotal role in the feedback regulation of the high-fidelity functioning of the photosystems and thus the security of the whole thylakoid system.

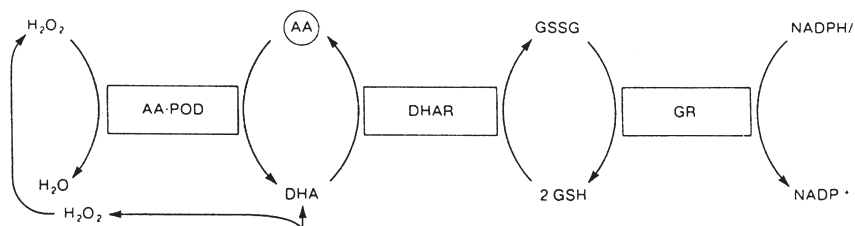


### Peroxide production during photorespiration

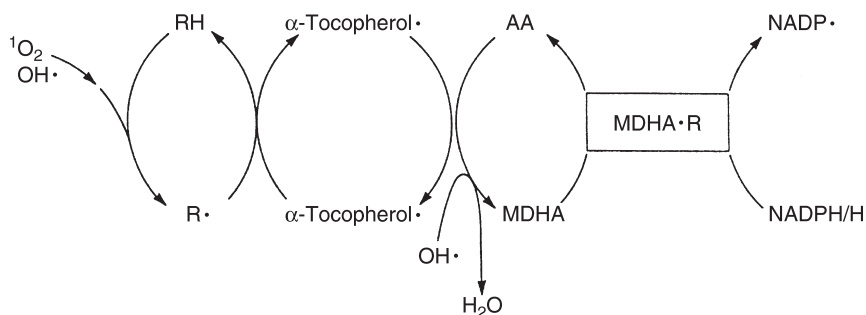


**Figure 3** Simplified scheme of photorespiratory processes. NADPH, reduced nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate; PGA, 3-phosphoglycerate.

8. In the same context, alpha-tocopherol molecules close to the P680 moiety of photosystem II (in proximity to carotenoids?) seems to play a pivotal role in the protection of the D1 shield protein from light-dependent damage via singlet oxygen. Tocopherol concentration in chloroplasts increases also in a light-dependent manner and during senescence. When tocopherol biosynthesis is blocked by a pyrazolinat herbicide that blocks 4-hydroxyphenylpyruvat (HPP) dioxygenase, i.e., the step in the biosynthesis between tyrosine and homogentisic acid, or by antisense mutants or deletion in prenylation steps, plants become extremely light-sensitive. Severe bleaching, degradation of D1 protein, and finally tissue necrosis are observed. This damage can also be prevented by the singlet oxygen quencher, diphenylamine (30). Thus, connecting this reaction with the ascorbate pathway (discussed previously) one can draw the picture (Fig. 5)



**Figure 4** Detoxification of H<sub>2</sub>O<sub>2</sub> in the chloroplast via ascorbate-glutathione. AA-POD, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; AA, ascorbate; GSH, reduced glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; GSSG, oxidized glutathione; NADP<sup>+</sup>, oxidized nicotinamide adenine dinucleotide phosphate.



**Figure 5** Singlet oxygen and OH-radical detoxification in the chloroplast. RH, reduced org. molecule such as fatty or amino acid; AA, ascorbate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MDHA, monodehydroascorbate; NADP<sup>+</sup>, oxidized nicotinamide adenine dinucleotide phosphate.

In addition, by induction of the key enzymes of the shikimate and mevalonate pathways, a wealth of antibiotic and/or antioxidative molecules belonging to several structural classes are de novo synthesized. Most of these molecules are thought to be involved in the overall strategy of plants' defense and are called phytoalexins; their inductions may be brought about by either abiotic (ozone, wounding) or biotic (pathogens) effectors, or by both (31,32).

Finally, photosynthetic processes, which are under metabolic and oxygen-detoxifying control, are converted into photodynamic reactions, which are controlled only by light and scavenger and/or quencher availability (phenolics and terpenoids, see late discussion). Photoinhibition may represent the threshold between these two basic, light-driven events.

This (more or less theoretical) sequence of events is not yet fully understood and in most cases can only punctually be characterized and followed by indicator reactions such as EPR (see Fig. 2).

In the following we concentrate on basic redox mechanisms during oxidative stress in plants.

### III. GENERAL MECHANISMS OF OXYGEN ACTIVATION

#### A. Rules and Pathways of Oxygen Activation in Plants

##### 1. How Are Free Radicals Generated? Homolytic and Heterolytic Reactions

During heterolytic reactions (Eq. [1]) electron pairs are transferred, either utilizing or creating ions:



Homolytic reactions (Eq. [2]) extinguish or create radicals through the transfer of single electrons, represented as a dot:

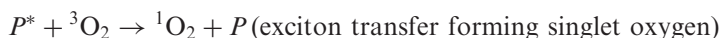


A radical is a compound containing an unpaired electron. There are stable and unstable radicals. Most free radicals are highly reactive, creating new radicals and thus initiating chain reactions. This holds especially for lipids in membranes.

Oxygen in the triplet ground state ( $^3O_2$ ) has to be activated in order to react with atoms or molecules in the “normal” singlet ground state, thus circumventing spin-forbidden reactions. The most important reactions of oxygen activation are briefly addressed in the following.

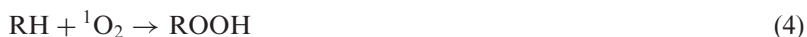
##### 2. Light Reactions

Oxygen can be activated by photodynamic reactions:



where  $P$  represents a pigment in its ground (singlet) state and  $P^*$  its activated triplet form, Eq. (3) represents a photodynamic reaction classified as photodynamic reaction type II in which singlet oxygen ( $^1O_2$ ) is formed.

In contrast to atmospheric oxygen,  $^1O_2$  is not subject to the spin rule and reacts rapidly with most organic molecules (RH), especially at double bonds, producing hydroperoxides:



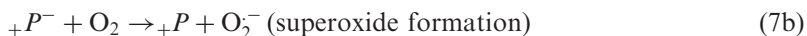
ROOH in turn can be reduced by one-electron donors ( $E^-$  representing reduced transition metal ions such as  $Fe^{2+}$  or  $Cu^+$ , semiquinones, heme and non heme proteins, isoalloxazines, or pteridines) yielding alkoxyl ( $RO\cdot$ ) radicals:



These  $RO\cdot$  radicals may initiate chain reactions, thus reacting with further co-oxidizing other molecules, for example by initiating co-oxidative bleaching of pigments:



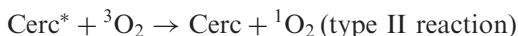
Photodynamic reactions undergoing charge separation within the excited pigment are called photodynamic reaction type I (where  $+P$  represents a photo-oxidized, i.e., bleached pigment):

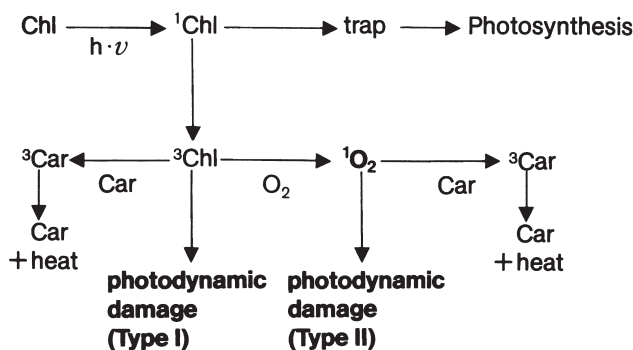


These light activated processes in the photosystems involving both protective and damaging components are summarized in Fig. 6.

### 3. Phototoxins

Production of ROS is observed after illumination of cercosporin, a perylene quinone toxin produced by several phytopathogenic *Cercospora* species, e.g., *C. beticola* and *C. kikuchii*. Cercosporin (Cerc) mainly seems to induce the formation of singlet oxygen and superoxide (33–35) in photodynamic reactions of both type I and type II, see Eqs. (3) and (7):





**Figure 6** Deactivation of excited states in the photosystems.  $^1\text{Chl}$ ,  $^3\text{Chl}$ ,  $^3\text{Car}$ ,  $^1\text{O}_2$  activated singlet ( $^1$ ) or triplet ( $^3$ ) states a, Photodynamic type I and type II processes; b, energy dissipation processes.

or

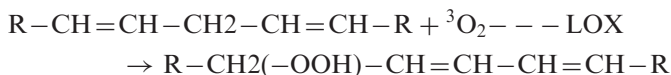


Similar light-dependent reactions are observed with other plant-derived products such as hypericins from St. John's wort (*Hypericum perforatum*) or several furanocoumarins from various Apiaceae (36).

Under illumination cercosporin induces lipid peroxidation in plant cells (37) followed by changes in membrane structure. Singlet oxygen quenchers such as diazabicyclooctane (DABCO) delay killing of cells by cercosporin. The formation of ROS by bacterial and fungal phytotoxins was reviewed in 1998 (38).

#### 4. Lipoxygenases Instead of Light

Equation 4 can also be catalyzed by lipoxygenase (LOX; see late discussion), utilizing triplet ground state oxygen instead of light where the peroxidation of unsaturated fatty acids yields conjugated diene hydroperoxides and "phyto-oxylipins" as messenger molecules such as jasmonic acid (39):



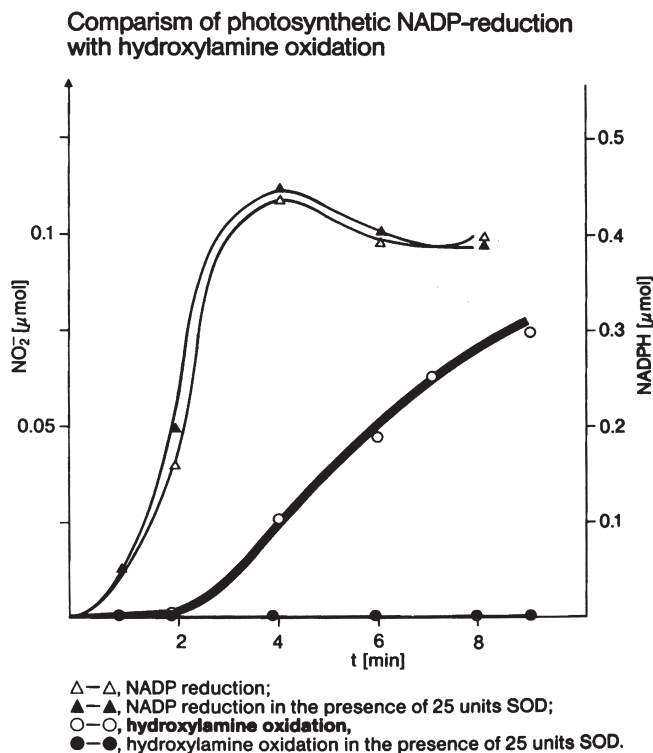
All the reactions can be measured by several methods and indicators in green plants if electron transport is impaired by poisons (herbicides, toxins) or other impact (40–42).

## B. Reductive Oxygen Activation

In the presence of appropriate reductants (see previous discussion; high affinity for oxygen; negative redox potential:  $E'_O$  of the redox pair  $O_2/O_2^- = -330\text{ mV}$ ; the reducing site of PSI has a redox potential of approximately  $-600\text{ mV}$  or even lower), superoxide may be formed from atmospheric oxygen:



Superoxide formation (measurable as hydroxylamine oxidation forming nitrite; inhibition by superoxide dismutase [SOD] by photosystem I) starts as soon as approximately 90% of the available (limiting!) NADP is reduced, as shown in Fig. 7.



**Figure 7** NADP reduction and superoxide formation by photosystem I. NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase.

Superoxide dismutates at neutral pH in aqueous media with a rate constant  $k = 2 \times 10^5 \text{ l} \cdot \text{M}^{-1} \text{ s}^{-1}$ , yielding hydrogen peroxide:



Similarly to Eq. (5), hydrogen peroxide may be reduced by certain electron donors, yielding the highly reactive hydroxyl radical ( $\cdot\text{OH}$ , redox potential close to +2V):



or



Equation (10a) is known as the Fenton reaction.  $\text{Fe}^{3+}$  can be reduced to  $\text{Fe}^{2+}$  by superoxide ion:



The sum of Eqs. (10a) and (11):



is known as Haber–Weiss reaction as recently reviewed (43).

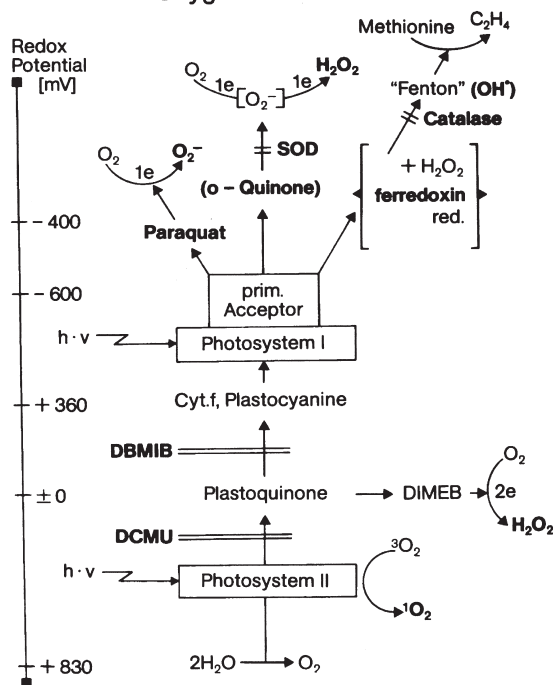
Altogether, one-electron transport reactions (superoxide and  $\text{OH}$  radical formation) catalyzed by ferredoxin or paraquat (herbicide) at photosystem I, “two-times one”-electron transport catalyzed by certain *o*-quinones such as dopamine or caffeic acid (SOD-inhibitable), or two-electron reductions by *p*-quinones such as dimethyl-methylendioxy-*p*-benzoquinone (DIMEB) at photosystem II can be differentiated by more or less specific inhibitors dibromothymoquinone [DBMIB], dichlorophenyl-dimethyl urea [DCMU] and indicator (detector) reactions, as outlined in Fig. 8.

Under in vivo conditions, the production and function of the free  $\text{OH}$  radical is doubtful (44) and “crypto  $\text{OH}$ ” (45) or metal-peroxide/peroxide–electron donor complexes have been proposed instead (46).

## 1. Hypohalides as Strong Oxidants

Another principle of the production of strong oxidants is the oxidation of halides ( $\text{X}^-$ ) by hydrogen peroxide producing hypohalites ( $\text{OX}^-$ ) catalyzed by certain peroxidases. Similar or analogous enzymes are operating both

### Sites and Products of Photosynthetic Oxygen Reduction

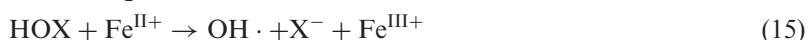


**Figure 8** Different electron outlets within the photosynthetic electron transport chain. SOD, superoxide dismutase; Cyt.f, Cytochrome, f; DBMIB, dibromothymoquinone; DIMEB, dimethyl-methylenedioxy-*p*-benzoquinone; DCMU, dichlorophenyl-dimethyl urea.

in the plant apoplast and in the neutrophilic phagosome as reviewed in Ref. 47.

These hypohalites in turn may be converted into  $\text{OH}^\bullet$  in the presence of superoxide or  $\text{Fe}^{\text{II}+}$ . Myeloperoxidases (MPOs) of neutrophilic and eosinophilic granulocytes catalyze the formation of hypohalic acids at the expense of hydrogen peroxide and chloride or bromide ions; the eosinophile peroxidase (EPO) is more active with bromide as compared to chloride.

The sum of the reactions discussed may be written as follows where  $\text{X}^-$  stands for the halide (48):

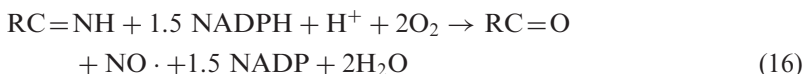




Equations (13) to (15) represent new reaction mechanisms unknown until very recently. Up to now, these reactions have only been described for white blood cells. We have recently found, however, that the apoplastic space of higher plants with its peroxidase content may operate similarly to the phagosome of leukocytes since plant (horseradish) peroxidase(s) catalyze(s) bromide- and hydrogen peroxide-dependent ethene release from ACC as an indicator for the formation of hypobromous acid. In contrast to bromide, chloride is completely inactive. Thus, plant PODs behave similarly to the eosinophile myeloperoxidase (49). We speculate that this reaction may be a relict from the “aqueous time” of all plants since there is enough bromine in marine water; chloride-dependent hypohalide formation was abandoned by apoplastic peroxides since it would have degraded ACC into ethylene in an unregulated manner, thus continuously inducing ethylene-linked hormone responses: senescence, organ abscissions, and so forth.

## 2. Peroxynitrite (ONOO<sup>-</sup>) as a Strong Oxidant Similar to OH-Radical or •OH

The simple molecule NO• is best known for its important role in the regulation of vascular tonus of animals (50), activating guanylate cyclase, thus producing the vasorelaxating cyclic guanosine monophosphate (cGMP). It functions as well in the central nervous system, where it regulates cerebral blood flow. NO is synthesized from the amino acid arginine (discussed later): activated leukocytes and endothelial cells produce NO (which should actually be written NO• since it is a free radical, just as nitrogen dioxide, NO<sub>2</sub>•) is from arginine catalyzed by the enzyme nitric oxide synthase (NOS; E.C.1.14.13.39) according to the following generalized formula:



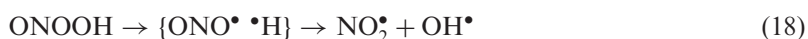
Alternative pathways of NO formation not involving arginine oxidation but the well-known nitrite reduction were proposed for plants in 2003 (51) and for animals in 1999 (52).

NO is also produced by plants during certain stress reactions such as senescence of pea foliage (51), in which its production controls leaf disk expansion and is correlated with ethene formation. In potatoes, NO initiates the formation of the phytoalexin rishitin and seems thus to be involved in plant “immunology” by analogy with animals (53).

Peroxynitrite ONOO<sup>-</sup> is formed from nitrogen monoxide and superoxide according to the reaction



Equation (17) is extremely fast and proceeds with a rate constant of  $k = 6.7 \times 10^9 \text{ n}^{-1} \text{ s}^{-1}$ , indicating that ONOOH formation is only diffusion-limited (see Refs. in 54 and 55). ONOOH is mainly produced under pathophysiological conditions (rapid superoxide formation together with NOS activation) in which the “cross-talk” between NO and other ROS seems to regulate both energy metabolism and pathogenicity (56). ONOOH initiates lipid peroxidation, inhibits mitochondrial electron transport, and inactivates glyceraldehyde-3-phosphate dehydrogenase as well as Na/K-ATPases and membrane sodium channels. This strong oxidant, which is produced in a slightly acidic medium ( $pK_a$  for ONOOH = 6.8) corresponds to the “free”  $\text{OH}^\bullet$ -radical formed (54) according to the following reaction:



Even if the percentage of free  $\text{OH}^\bullet$  (relative to that of other radicals generated as calculated by electron paramagnetic resonance-results) seems to be as small as only a few percent of total ONOOH the effect may again be “site specificity” due to the “cage”  $\{\text{ONO}^\bullet \cdot \text{OH}\}$ , and especially to the “escaping”  $\text{OH}^\bullet$  in the immediate proximity of the locus of formation. In model reactions, both hydroxylations and nitrations of phenolic compounds have been reported. Plants seem to be to some extent protected against such nitrations by endogenous scavenger molecules: tyrosine nitration by ONOOH is strongly inhibited by several plant extracts (*Populus* sp., *Fraxinus* sp., leaf and bark extracts) containing high amounts of such polyphenols (57). One may summarize NO and peroxynitrite biochemical reactions by the question, Is nitric oxide toxic or protective? The protective and cellular antioxidant inducing the signaling and the toxic properties of NO thus render it a Janus-faced molecule (58). Nevertheless, the “cross-talk between NO and oxyradicals” represents “a supersystem that regulates energy metabolism and survival of animals” (56) and plants (59). Altogether we have to deal with the radical and nonradical type ROS indicated in Table 1.

All these ROS interact with each other in a very complex manner (for example, at sites of inflammation in animals or wounding in plants) so that one has to envisage most of the species discussed at the same time and the same place. Therefore, all types of important biomolecules may be in the focus of attacks by them.

### C. Compartmentalization of Oxygen Activation

All compartments of cells possess enzymic and nonenzymic tools for oxygen activation, some only after exogenous initiation such as wounding, infection, UV radiation, or poisoning. The basic mechanisms may again be divided into light and dark reactions. Transition metal-dependent

**Table 1** The Most Important Reactive Oxygen Species

## Free radicals

- Atmospheric oxygen,  $O_2$  (diradical)
- Superoxide radical anion,  $O_2^{\cdot-}$
- Hydroperoxyl radical,  $HO_2^{\cdot}$
- OH radical,  $OH^{\cdot}$
- Alkyl and alkoxyl radicals,  $R^{\cdot}$ ,  $RO^{\cdot}$
- Peroxyl radicals,  $ROO^{\cdot}$
- Nitrogen monoxide or dioxide,  $NO^{\cdot}$  and  $NO_2^{\cdot}$

## Nonradical compounds

- Hydrogen peroxide,  $H_2O_2$
- Organic peroxides,  $ROOH$
- Hypohalous acids or their salts, such as  $HOCl$ ,  $HOBr$ , and organic chloramines such as taurine chloramine
- Peroxynitrite,  $ONOOH$
- singlet oxygen,  $^1O_2$

reactions involving activated oxygen mainly concern iron and copper ions (43). Iron chelates appear to dominate in this type of activating reaction, and lipid peroxidation is one of the most representative chemical symptoms.

## 1. Mitochondria

Mitochondria represent an important site of oxygen activation that forms superoxide and successors. Under stress conditions, aging, fruit ripening, an alternative pathway (alternative oxidase, [AO]) that does not include the cyanide-sensitive cytochrome c/a pathway may be induced, one that in plants (in contrast to animals?) apparently does not include the production of ROS (60,61). This alternative way seems to be strictly correlated and induced by the internal peroxide level and is counteracted by tocopherol (62). The expression of the AO seems to be accompanied by the expression of an uncoupling protein (which abolishes the proton gradient formed during electron transport) (63), thus increasing electron flow from endogenous electron donors and lowering the reduction charge.

Superoxide and peroxide in animal mitochondria are formed at, or close to, reaction complexes I and II, i.e., in the proximity of the ubiquinol–flavoprotein–nonheme iron complex oxidizing NADH and/or by cytochrome b (64). Cytochrome c peroxidase and Mn SOD seem to detoxify reactive oxygen species cooperatively. Mitochondria by means of their glycine decarboxylase system are mainly involved in C-1 metabolism, which plays an important role in stress metabolism (methylation of phenolics; see Ref. 61).

## 2. Microsomes

Microsomes are artifactual preparations derived from the endoplasmic reticulum that represent an electron transport system reductively activating the heme iron protein cytochrome  $P_{450}$  at the expense of NAD(P)H via flavin-dependent reductases, non heme iron “redoxins,” and/or a b-type cytochrome. After inductions by several intrinsic or xenobiotic metabolites, this electron transport system may cause peroxidations by an “overshoot” phenomenon, attacking its own membrane lipids after cessation of its hydroxylating task, mediated by superoxide and its derivatives. Several anticancer drugs (daunorubicin and doxorubicin; Adriamycin) have been shown to bind to this reductase, thus initiating redox cycling under production of ROS (see refs. in Ref. 25).

## 3. Peroxisomes

Peroxisomes, in contrast to mitochondria, are compartments with a single unit membrane, containing xanthine oxidase as  $O_2^-$  and  $H_2O_2$ -producing enzyme in addition to glycollate oxidase and fatty acid- $\beta$ -oxidases as  $H_2O_2$ -producing enzymes and NOS; high concentrations of catalase and peroxidase in addition to SOD are involved in detoxification of reactive oxygen species. Peroxisomes are mainly involved in photorespiration; production of ROS and NO are speculated to be involved in redox-based signaling (65).

## 4. Plasmatic Membranes

Plasmatic membranes contain NAD(P)H oxidases that are more or less specific for their electron acceptors, acting similarly to diaphorases (66,67). Under certain circumstances, these flavoproteins may be rendered auto-oxidizable as shown for hypersensitive responses in plants after infections. Under these conditions superoxide and its derivatives that produced again may function as cellular messengers. The most prominent NADPH oxidase is the one being established in the membranes of leukocytes and in the plasmalemma of plant cells and finally responsible for the “respiratory burst” producing superoxide after appropriate activation, for example, by pathogens (68).

## 5. Cell Walls

Plant cells, in contrast to animal cells, contain more or less rigid cell walls that, together with the intercellular space, comprise the apoplastic

compartment. Oxygen activation in this extracellular space includes both products derived from the NAD(P)H oxidase of the plasma membranes and the products of cell wall intrinsic enzymes such as peroxidase(s) (69). These peroxidases produce hydrogen peroxide at the expense of NADPH in a manganese-catalyzed reaction, allowing an extremely complex crosslinkage of C6–C3 phenylpropanoids forming lignine (70) as an essential element of woody plants and a component of pathogen defense.

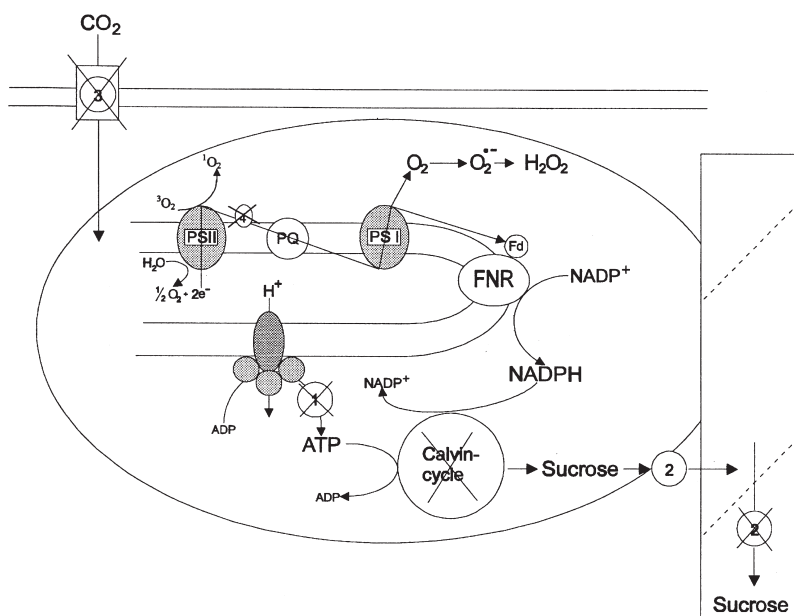
#### **D. Phytopathological Aspects**

Stress in plants differs from that in animals or humans. Fundamentally stress is coupled with signal feedback between different sensors and organs. The term stress is used for the description of processes and symptoms during which the individuum is supposed to be in a more or less critical situation or to suffer. Thus, it is extremely difficult, if not impossible, to define thresholds between normal metabolism and stress metabolism exactly.

Several authors and editors (Ref. 71) have reported on ecological factors and developmental processes connecting stress and oxygen activation. Wounding or other mechanical impacts influence transport across membranes or in the apoplast by increasing metabolic feedback to the photosystems, thus inducing oxidative processes. Fatty acid peroxidation through decompartmentalization; ionizing or UV radiation; drought; flooding; osmotic impacts (high salt concentrations, desiccation); deficiency in macro- or micronutrients; dramatic temperature changes such as heating, chilling, or freezing; as well as poisoning (air pollution, water and soil pollution, herbicide treatment) cause changes in pro- and antioxidative potentials and generate a response of hormone synthesis or release, which in turn may increase resistance, stability, avoidance, tolerance, or repair (see earlier discussion).

Again, the most important concept is that all these impacts underlie feedback to the chloroplast: independent of the site of transport or metabolic “block,” whether in the roots (salt, drought, mineral deficiency), in the transport system (xylem or phloem blocks by infections), in phloem loading (photo-oxidants, infections), in limitations in CO<sub>2</sub> fixation (stomatal closure, lack of Calvin cycle activities due to enzyme inhibition), or in photosynthetic electron transport itself. As a consequence photosynthetic oxygen activation and/or photodynamic processes are initiated, leading to a chain of reactions as outlined in Refs. 14 and 15 and Fig. 9.

An important field of research on oxygen activation during host–pathogen interactions is focused on reaction mechanisms of toxins produced by pathogenic fungi and bacteria. Several toxins have been shown to act as redox cyclers or act photodynamically, i.e., through the light-dependent



**Figure 9** The metabolic feedback chain in plants initiating oxidative stress. PQ, FNR,  $\text{NADP}^+$  oxidized nicotinamide dinucleotide phosphate; NADPH, reduced nicotinamide dinucleotide phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

formation of activated oxygen. In this context the quinoid derivatives cercosporin, dothistromin, and dihydrofusarubin have to be mentioned (see previous discussion and Ref. 72). In other cases bacterial or fungal toxins introduced into the plants react by analogy with certain herbicides such as the “quat” dyes: In 1998 Heiser et al. documented (38), naphthazarin toxins such as dihydrofusarubin produced by certain strains of *Fusarium solani* induce redox cycling and superoxide production with similar kinetic properties to those of methylviologen after reduction by photosystem I or by certain NAD(P)H oxidoreductases (such as diaphorases). These redox reactions are responsible for bleaching and necrotization of the treated plants. All oxygen-activating phytotoxins have been shown to include both photodynamic and reductive mechanisms, as reported in Chapter 11.

### 1. Roles of Phenolics as Metabolic Regulators and Antioxidants

Phenolics are ubiquitously found in the plant kingdom. Among the approximately 50,000 “secondary” plant metabolites phenolics represent the largest group. Many of them are thought to be relicts of phylogenetic

origin with lost functions. Today we know that phenolics contribute to the overall fitness of plants (73) with known multifold functions, such as insect attraction by colors and protection against pathogens or competitive neighbor plants, to mention just two (74). Since phenolics are composed of one or more benzene rings containing various substituents, especially hydroxyl groups, their biochemical activities are extremely broad-ranged. Depending on the neighboring substituents of a phenolic hydroxyl group, its antioxidative properties are outstanding and include all known mechanisms. This is of special importance not only for plant themselves but also for humans and animals, in which they act as vitamins and/or protectants against oxidative stress (see discussion later and Refs. 75–77).

Phenolic compounds not only play an important role in plant resistance and defense against microbial infections, which are intimately connected with ROS, they may also act as chelators for iron or copper ions (see later discussion). Likewise sequestration of essential microelements such as molybdenum may be mediated by phenolics such as anthocyanins (78).

Principally there are two types of phenolics found in plants: those that are constitutively present (“preformed” defense molecules) and those that are newly synthesized after an eliciting process, e.g., after infection. During and after infections, *de novo* synthesis of phenolics as well as phenol-oxidizing enzymes (phenol oxidases, peroxidases) plays a pivotal role in defense in at least a three ways:

1. Their function in wound healing and active defense involves the formation of barriers by melanin-type polymers by phenol-oxidases and superoxide production by a cooperative phenolase–photosystem I–dependent redox cycling of *o*-dihydroxy phenylpropanes such as dihydroxy phenylalanine (DOPA) or dopamine.
2. The formation of phytoalexins from basic phenylpropanes such as cinnamic acid or phenylalanine and subsequent hydroxylation(s), finally yielding polyphenols and the corresponding quinones; during the induction of these metabolic processes, elicitor-induced production of reactive oxygen species including different host–parasite interactions, has been reported by several groups. During these elicitor-induced effects superoxide dismutase–inhibitable cytochrome c reduction and lipid peroxidation can be measured.
3. The action of *o*-quinones as  $\text{NH}_2$  or SH acceptors via a Michael-type addition, thus inactivating vital functions of invading microorganisms.

When phenol metabolism is impaired, for example, by *Helminthosporium oryzae* toxin, part of the plant resistance against the pathogen is lost. These few examples may suffice to demonstrate that there are a few universal schemes underlying most stress effects in plants.

## 2. Lipid Metabolism During Stress

Membrane lipids, especially phospholipids, play a pivotal role in plant growth, development, and response to environmental stress, in which phospholipases (79) frequently catalyze the first step in the release of signal molecules such as phosphatidic acid (as phosphatidylinositol di- or triphosphates, 80) and diacylglycerol, which in turn trigger calcium signals or protein kinases. Phosphatidic acid as one phospholipase D product in turn has been shown to induce the generation of superoxide by NADPH oxidase (81).

Unsaturated fatty acids in such membrane lipids are frequently the target of an early attack by ROS in which the corresponding hydroperoxides are formed. In storage lipids these hydroperoxides are formed via 13-lipoxygenases and degraded by a phospholipase and a triacylglycerol lipase, representing a novel pathway of fat degradation during germination (82). In order to prevent more or less “chaotic” and aldehyde-producing decay of these hydroperoxides, alkylhydroperoxide reductases (phosphatidyl hydroperoxide-glutathione peroxidase; peroxiredoxin) may function, thus partially preventing radical chain reactions and Amadori chemical reactions (83).

In animal metabolism arachidonic acid is converted into several hormonelike metabolites such as prostaglandins and leukotrienes that operate as messengers during and after infections. In plants “phyto-oxylinins” are produced by a set of enzymatic processes from linoleic acid (C18:2) or linolenic acid (C18:3) catalyzed by lipoxygenases either inserting a perhydroxyl group at C-9 (9-LOX) or at C-13 (13-LOX). After further derivatization a wealth of compounds (hydroxides, aldehydes, ketols, ketones, cyclized compounds, divinyl ethers, epoxy alcohols), which are thought to cooperate and interact in the concert of regulation and organization of defense, are obtained (39).

## 3. Lipid Peroxidation and Induction of Chlorosis and Necroses by Reactive Oxygen Species

Oxidative processes such as lipid peroxidation occur if ROS are produced at a cellular level and the detoxification system is overloaded or exhausted. The peroxidation of unsaturated fatty acids is induced by hydroperoxy radicals ( $\text{HO}_2^\bullet$ ) or by OH radicals or electron donor- $\text{H}_2\text{O}_2$  complexes



("crypto-OH"; see also peroxyxynitrite, Eq. [18], and Refs. 45, and 46), primarily yielding lipid radicals via abstraction of a hydrogen atom. Singlet oxygen directly produces lipid hydroperoxides by analogy with Eq. (4) (see also Sec. III. 3).

Alkoxy radicals ( $\text{LO}^\bullet$ ) from this process can attack pigments such as chlorophyll (CHL) or carotenes that are oxidized (bleached) by "co-oxidation" (72).



#### IV. SIGNAL TRANSDUCTION CHAINS AFTER INFECTIONS

Transmembrane signaling during elicitor recognition and induction of resistance or hypersensitive response in plants has become one important focus of recent research in molecular phytopathology (84). Elicitor recognition, superoxide formation and signal transduction in plant defense seem to involve guanosine triphosphate-(GTP)-binding proteins as well as  $\text{Ca}^{2+}$ -dependent protein phosphorylations, similar to activated leukocytes. After binding of GTP to the 45kd protein, a rapid oxidative burst is observed. Elicitor-dependent activation of a  $\text{Ca}^{2+}$ -dependent protein kinase yields a transient release of hydrogen peroxide. There is a relationship between induction of resistance against pathogens and induction of pathogenesis-related proteins (PR proteins) and salicylic acid (85–87). There are also salicylate-independent pathways of resistance induction (88). In many cases induction of disease resistance and/or hypersensitive (necrotic) cell death is dependent on hydrogen peroxide and NO.

Wounding-induced increase of jasmonic acid operating as one "master switch" in resistance induction (89) and in induction of the respiratory burst seems to proceed via different signaling pathways, which can be parallel, cooperative, or/and inhibitory; these interactions are not clear (90,91). At the end of these signaling and resistance-inducing pathways production of plant "antibiotics" (phytoalexins) contributes to the overall, broad-based plant defense system.

Phytoalexins comprise a wide range of low-molecular weight substances from different classes of so-called secondary plant substances such as flavonoids, isoflavonoids, pterocarpanes, stilbenes, terpenoids, and coumarins (31,32).

With increasing knowledge we have to realize, however, that primary (i.e., membrane-located) signal transduction pathways after pathogen recognition or stimulation are quite similar in plants and animals. The responses after the recognition and signaling processes in plants concern a

wealth of different interconnected networks that communicate with each other via hormones (92), including light-dependent (phytochrome, cryptochrome) and gaseous (ethylene, methylsalicylic acid) signals, allowing even interorganismic communications.

In plants, however, in contrast to animals, pathogen defense is connected with a unique expression of genes allowing an incredible plasticity and flexibility of metabolic responses based on their synthesizing capacity (93). Another similarity between animals and plants is located on the level of amine transmitters: aromatic monoamine oxidases, as in animal neuronal systems, are also involved in plant signaling inducing calcium fluxes across plasmatic membranes (94).

## **V. OXYGEN ACTIVATION AND FUNCTION OF ANTIOXIDANTS IN HUMAN AND ANIMAL DISEASES: MAIN MECHANISMS AND PRODUCTS IN COMPARISON TO THOSE IN PLANTS**

Animals can principally produce the same ROS as plants, except reactions observed in chloroplasts. The mechanisms of oxygen activation are identical or analogous.

The most prominent and generally toxic ROS are produced after infections and during inflammations, represented by the following reactions:

1. XOD reaction and respiratory burst, producing OH radicals and Fenton-type oxidants, especially  $\text{Fe}^{2+}\text{-H}_2\text{O}_2$ .
2. Hypochlorite,  $\text{OCl}^-$  (via myeloperoxidase reaction after degranulation); the reaction between superoxide and HOCl seems also to produce OH radicals (see earlier discussion).
3.  $\text{NO} + \text{superoxide}$  (both simultaneously derived from activated leukocytes or endothelial cells or by the drug molsidomin [SIN-1]) forming peroxynitrite,  $\text{ONOO}^-$ .

These species have recently been tested as to their destructive potentials to more or less specific biological indicator molecules for ROS and their protection by plant ingredients (49,57).

### **A. Protection from Oxygen Stress and Oxygen Detoxification**

Since activated oxygen is toxic, it has to be under continuous strict control of integral detoxification processes, detoxifying enzymes, and organic

antioxidants. One principal way to deal with oxygen toxicity is avoidance, i.e., circumventing of one- or two-electron donating processes toward oxygen. This can be achieved by “tight” coupling of electron transport chains operating at the electronegative region of oxygen activation or by stoichiometric coupling of oxygen activating processes with utilization of activated oxygen. Another possibility is the inhibition or inactivation of oxygen activating processes or enzymes. This has been shown for xanthine oxidase, lipoxygenases, prostaglandin cyclase, NAD(P)H oxidases and other enzymes by a wealth of compounds used in medicine. The so-called nonsteroidal anti-inflammatory drugs (NSAIDs) and several flavonoids are good examples of this principle, which is considered in another chapter.

## B. Integral Detoxification Processes

Integral detoxification processes (also see earlier discussion) connect elementary reactions of intermediary metabolism with detoxification of reactive oxygen species. This may be achieved

1. By activation of enzymes or induction of isoenzymes such as peroxidases, DT diaphorase or members of the P<sub>450</sub> group.
2. Through coupling of peroxide utilization with NADPH oxidation via ascorbate peroxidase, ascorbate and glutathione reductase.
3. By the “peroxidized membrane repair team,” including phospholipase(s) and glutathione peroxidase, thus in concert opening membraneous positions of peroxidized fatty acids for renewing activities.

Phenolic compounds play an important role in this context, acting as antioxidants, inducers of enzymes, transition metal chelators, thus avoiding Haber–Weiss–(Fenton)chemical processes; and cofactors of regulation of enzymic activities.

Detoxification in a wider sense thus also concerns the replacement of damaged molecules such as deoxyribonucleic acid (DNA), proteins, and membrane lipids by a complex “crew” of integrated repair enzymes and replacement processes. Continuous involvement of these repair processes, however, would render them inactive since they also continuously function as targets of these reactive oxidants. Therefore, another batch of first-aid molecules such as phenolics is biologically more than logical: the only “help” for the final repair teams is small molecules with “kamikaze-type” properties, representing antioxidants with or without a chance of being metabolically repaired themselves.

## 1. Detoxifying Enzymes

As already mentioned, detoxification by enzymic processes is only possible if the reactivity of the respective oxygen species is reasonably low under physiological conditions so that the enzymic reaction allows at least one to three orders of magnitude between the reaction under enzyme catalysis and the noncatalyzed spontaneous reaction between the oxygen species and any reaction partner in its “molecular neighborhood.” Therefore, the reactions of  $\text{OH}^\bullet$ ,  $^1\text{O}_2$ ,  $\text{RO}^\bullet$ ,  $\text{ROO}^\bullet$ , and  $\text{HOO}^\bullet$  are not under enzymic control; their reaction constants with potential reaction partners in their typical environments are too fast (generally  $k \gg 10^8$ ) for enzyme catalysis. Thus, the reactions of biomolecules with these oxygen species have to be amended after damage. In order not to flood these repair process the antioxidative molecules mentioned serve as scavengers and quenchers of activated states.

Enzyme-catalyzed detoxifications mainly concern superoxide, peroxides, and epoxides (produced by cytochrome  $\text{P}_{450}$  activities) as more or less stable reduced oxygen species.

In most aerobic cells catalase (CAT), superoxide dismutases (SODs), ascorbate peroxidase, mono- or dehydroascorbate reductases, glutathione peroxidase (GSH-POD), glutathione reductase, and different peroxidases (PODs) either individually or cooperatively remove stable reactive oxygen species. Different individual physiological parameters or “stresses” may induce different enzymes. For example, exposure of humans to hyperbaric oxygen (HBO) causes DNA damage in lymphocytes only after the first treatment and not after further treatments of the same individual. Induction of heme oxygenase 1 (HO-1) in lymphocytes and thus adaptive protection against peroxide toxicity (via sequestration of iron) has been shown. The enzymes SOD, catalase, endonuclease, and DNA polymerase  $\beta$  remained unchanged after HBO (95).

## 2. Phenolic Derivatives in Protection from Oxidative Stress

Phenolic redox reactions are fundamentally involved in stress metabolism both in plants and in animals, comprising redox processes and antioxidative functions including the formation of phenoxyl radicals, semiquinone radicals, and *o*- or *p*-quinones undergoing electron donating reactions to reactive radicals. Depending on the neighborhood the formed phenolic radical may be rather stable, awaiting reduction by available electron donors such as ascorbate and  $\alpha$ -tocopherol. In a “pecking order” (96) of these two important antioxidants, radical states in biomembranes are quenched where ascorbate or thiols such as reduced glutathione or lipoic acid (thioctic acid) regenerate the reduced state of phenolics such as

tocopherol or ubiquinol in the interphase between lipophilic and hydrophilic plasmatic phases (97,98).

With certain initiator radicals phenolics may be converted into alkoxy radicals ( $\text{RO}^\bullet$ ) or semiquinones, thus acting as pro-oxidants, depending on the substituents in the neighborhood of the phenoxyl radical group; tocopherols acting as pro-oxidants are good examples of this process. In the presence of ubiquinol, however, the pro-oxidative activity of vitamin E is converted into an antioxidative function, as shown for low-density lipoprotein (LDL) oxidation (99). Thus cooperative effects of diverse phenolics are indicated in which the over all antioxidative effect is due to "total phenolics" and not a single substance and additive, synergistic, and supplementary effects are observed. In the case of transition metal catalysis (Fenton or Haber–Weiss chemical reactions), phenolics may act as chelators for iron or copper ions. In this respect they may either stimulate or inhibit oxidative reactions, their effect is strongly dependent on the model reaction or the type of damage observed. Phenolics may simply act as radical scavengers or radical-chain breakers, thus extinguishing strongly oxidative free radicals such as  $\text{OH}^\bullet$ ; they also may react with nonradical species such as hypochlorous acid or  $\text{ONOOH}$ , yielding products with much lower oxidative capacities than the parent compounds (75,76,100,101).

### 3. Modulation of Enzyme Activities by Phenolics

As mentioned the strong oxidant  $\text{ONOOH}$  is produced from NO and superoxide. Thus, under pro-oxidative conditions such as activation of xanthine dehydrogenase rendering its activity an oxidase (XOD), NO (measured as nitrite,  $\text{NO}_2^-$ ) is produced from hydroxylamine. This reaction is completely inhibited by SOD and strongly stimulated by myoglobin. We have shown that in the presence of XOD, xanthine (or acetaldehyde; alcoholism!) and hydroxylamine,  $\text{NO}_2^-$  formation, as an indicator for intermediary NO, is completely abolished by 100 U of SOD. Addition of  $10^{-5}$  M myoglobin instead of 100 U SOD doubles nitrite formation from hydroxylamine. In the presence of both myoglobin and SOD, nitrite formation is unaffected by SOD. Thus myoglobin renders the protecting enzyme, SOD, inactive. Both the flavonoids, quercetin and rutin, however, inhibit nitrite formation by the XOD system similarly to inhibition by SOD even in the presence of myoglobin. This function is not due to dismutation of, or reaction with, superoxide, but to inhibition of XOD activity. Likewise, MPO activity is inhibited by quercetin or by rutin (see refs. Ref. 102).

In contrast, NO formation by aorta endothelia seems to be enhanced by red wine polyphenolic compounds such as leukocyanidol but not by catechol as defined by blood vessel relaxation, electron paramagnetic

resonance (EPR) spectroscopy, and increase of cyclic guanosine monophosphate (GMP) (103). Thus certain polyphenolics very specifically induce vasorelaxation by enhancing reactions involved in NO production by endothelia and not by superoxide scavenging. Others interfere with enzymes (XOD, MPO), produce ROS, thus ameliorating possibly damaging processes.

Some molecules such as quercetin (QU) seem to have just one more function: quercetin has been shown to potentiate antioxidative (scavenger) functions as well as iron chelating and enzyme-inhibitor properties (see earlier discussion). In 2001, Fiorani and associates (104) reported on the prevention of dehydroascorbate (DHA)-dependent GSH depletion in red blood cells, which was prevented in the presence of quercetin. The mechanism was not simply chemical interaction of QU with DHA or oxidized glutathione (GSSG), but activation of enzymic GSSG reduction downstream of this primary redox event.

#### 4. Allelopathic Factors, Essential Oils, and Terpenoids

Essential oil (EO) derived from steam distillation of needles and bark of *Pinus* and *Picea* species and also from a plethora of other plant sources is widely used in ointments, bathing oils, and inhalant drugs for curing a wide range of bronchial, skin, and muscle disorders of infectious, rheumatic, or neuralgic origin. Oleoresins containing these oils comprise various amounts of monoterpenes (10 C atoms) such as  $\alpha$ - and  $\beta$ -pinene,  $\Delta^3$ -carene, limonene, terpinolene,  $\gamma$ -terpinene, and myrcene as major components. In addition to these monoterpenes, sesquiterpenes (15 C atoms) and diterpenes, containing 20 C atoms, are known. Volatile and resin-bound terpenoids are thought to represent a broad defense system allowing protection of conifers against pine weevils, bark beetles, and associated fungal pathogens. Antioxidative properties of essential oils from eucalyptus, myrtle, lemon, and pine were reported since 2001 (4,105,106).

The compartments of highest concentration of essential oils (EOs) in the plant are special oil cells, which are eventually converted into oil containers. Plant families containing EOs are abundant among the Apiaceae, Asteraceae, Cupressaceae, Laminaceae, Myrtaceae, Pinaceae, Poaceae, and Rutaceae. In 2002, the biosynthesis of terpenoid resins and resin duct induction in developing Norway spruce xylem has been described and the whole field was reviewed intensively (107). The authors discuss induction of biosynthesis by mechanical influences after insect attack, fungal infections, and methyl jasmonate treatment.

There are good reasons to assume ecological functions for most EOs, such as acting as allelopathic agents, repellents or attractants in plant-plant

or plant–pathogen/herbivore interactions since their biosynthesis is induced by insect feeding, eventually triggered by an elicitor from oral secretion of the feeding insect (107).

The EOs are in a liquid state at ambient temperatures, almost colorless and optically active. They are soluble in most organic solvents, but their water solubility is very limited.

The biosynthesis of monoterpenes (MTs) proceeds via the well-known mevalonic acid pathway starting from acetyl coenzyme A (CoA) by various condensations including activations by ATP and reductions by NADH. A key step is the two-step reduction of hydroxymethyl-glutaryl CoA (HMGCoA) into mevalonate under CoA release by the enzyme HMGCoA reductase.

In 2002 another pathway, not from acetyl CoA but from pyruvate and glyceraldehyde-3-phosphate (GAP), was established for plastidic-derived terpene derivatives in the mid-1990s, especially by the *Lichtenthaler*-group (Refs. in Ref. 107). This pathway, in contrast to the mevalonate pathway, is not inhibited by HMGCoA R inhibitors. It includes, after a thiamine-dependent condensation of pyruvate and GAP (CO<sub>2</sub> release!), 1-deoxy-D-xylulose-5-phosphate (DOXP), 2-C-methyl-D-erythrose-P, and 2-C-methyl-D-erythritol-4-P as precursors of isopentenyl-pyrophosphate (IPP).

The MTs are widely distributed and contribute sometimes more than 80% of the total oil content.

The basic structure of the monoterpene skeleton is cyclic menthane, but there also exist acyclic and irregular members such as myrcene and ocimene or artemisiaketone. The MT hydrocarbons comprise important compounds present in various essential oil compartments of different plant groups and include well-known structures such as pinene, limonene, and terpinene. These compounds are frequently found as isomers, for example,  $\alpha$ - and  $\gamma$ -terpinene, as optical isomers, (+)- and (–)-limonene or both, (+)- $\alpha$ -phellandrene and (–)- $\beta$ -phellandrene, or (+)- and (–)- $\alpha$ -pinene together with (–)- $\beta$ -pinene.

Essential oils also may contain degradation products of membranous unsaturated fatty acids such as “green odor” components (*cis*- or *trans*-hexenal, hexanal, and hexanol) and several lactones or peroxides. Furthermore, essential oils contain compounds from terpene degradation, for example, C<sub>13</sub>-norisoprenoids and sulfur- and nitrogen-containing molecules (pyridine derivatives in spearmint oils). Several volatile terpenoids are present in plants as glycosides and contribute to the overall, typical fruit flavors.

Another function of EOs is seen in defense and wound sealing in pine tree species in which a coordinated induction of the biosynthesis of MT and resin acids is observed. The MTs are thought to function as solvents and



vehicles for resin acids, as the MTs evaporate at the wound region and resin acids undergo oxidative polymerization and crystallization in this very region, thus preventing penetration sites for pathogens.

Plants at extreme sites often have to tolerate extreme temperatures. The direct correlation between isoprene concentration and thermotolerance led to the speculation that these compounds increase thermotolerance by interacting with membranes of the plants or protecting against singlet oxygen toxicity (108). Such an interaction is also assumed for physiological and pharmacological functions in humans.

## 5. Pharmaceutical Values and Toxicities

Essential oils exhibit antiseptic (to viruses, bacteria, and fungi), sedative, spasmolytic, bronchiolytic, and also irritating properties. There are toxic oils, such as boldo, chenopodium, thuja, and mustard, and toxic oil ingredients, such as thujones, carvone, carvacrole, and pulegone, but fortunately acute toxicity of oral uptake, especially among those mainly used, is generally very low, with a lethal dose in 50% ( $LD_{50}$ ) of 2–5 mg/kg or above. If they are taken up erroneously in high quantities (by children) intoxication and even death has been observed. This holds especially for camphor ( $LD_{50}$  = ca. 1.5 mg/kg), which causes epileptic convulsions, and for EOs from plants such as clove, wintergreen, and parsley. There are some observations concerning skin toxicity (irritations, sensitization after topical applications, e.g., by cosmetics or perfumes), including phototoxicity (e.g., photodynamic compounds in the Apiaceae = Umbelliferae family). Allergic reactions provoked by EO application are mainly due to the formation of isomeres, cyclic compounds, or peroxides during storage under inadequate conditions such as high temperature, light, and access of air (36).

Beside the properties mentioned (antiseptic, spasmolytic, sedative) in classical medicine EO therapy mainly concern inflammatory processes (IPs) of skin, gastro intestinal tract, bronchial tract, and the joints. As far as the bronchial tract is concerned the benefits of EOs have been pointed out, although certain restrictions as to the therapeutic range have been discussed.

The mechanisms of EOs in attenuation of IP is still under debate however, hypothesized influence on the induction and perpetuation of the production of reactive oxygen species (ROS) via different reactions during the inflammatory process is gaining support. In addition to these antioxidative effects, EOs (for example, from *Protium heptaphyllum*) seem to inhibit leukocyte accumulation and attenuate NO metabolism and



leukotriene (LTC<sub>4</sub>) production by human basophils and eosinophils, thus exhibiting properties at the regulatory site of inflammatory processes.

## **VI. EXPLOITING THE PLANT'S SYNTHESIZING CAPACITIES: DRUGS AND FUNCTIONAL FOOD**

### **A. Pharmacognosy and Functional Food**

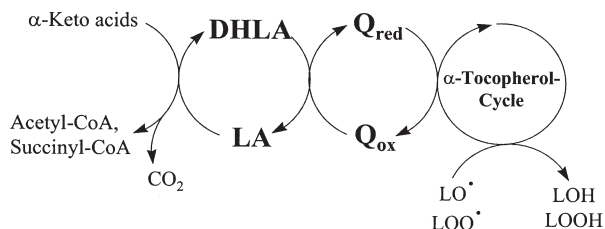
As outlined, an extremely complicated network of pathways provoked by oxidative stress is involved in cross-tolerance (109).

Diverse groups of plant ingredients produced by stress or constitutively present have been used traditionally as either medicine or poisons. In the past and particularly in the last decade, they have been commercially exploited as food ingredients and proposed as prophylactics in disease prevention. Extensive publication about these applications has occurred in the past dozen years.

In the present, envisioning an ever growing population of elderly people, neuronal disorders (Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, mental depressions), and heart-blood circulation problems (atherosclerosis, stroke), and cancer are known to represent major disease groups spreading worldwide. Therefore, research is concentrating on these topics in a steadily increasing number of publications. The sections that follow discuss a few of them.

#### **1. Atherosclerosis**

Low density lipoprotein oxidation is thought to be one initiating factor in atherogenesis. There have been numerous publications in the past 10–15 years reporting on prevention of LDL oxidation by a “pecking order” principle (96,99) by food supplements, genistein (110), cocoa (111), and many others. Besides the well-known cooperative “repair teams” tocopherol–ascorbate and tocopherol–ubiquinol–dihydrolipoic acid (see refs. in Ref. 98), genistein (in soybean products), and cocoa (in dark chocolate) have been suggested to be helpful as preventive “food-drugs”. Another “lipid-protecting team” in LDL, involving intrinsic carotenoids ( $\beta$ -carotene, lycopene), may be operating: carotenoid oxidation was strongly delayed by the lemon oil terpene,  $\gamma$ -terpinene (4,106), in a similar manner to that of tocopherol by ubiquinol. In this case, the oxidized quinones are reduced by alpha keto acids (NADPH) via the diaphorase-thiocitic acid (= lipoic acid) pathway, as shown schematically in Fig. 10.



**Figure 10** Detoxification of peroxyl and alkoxyl radicals by the cooperative tocopherol-ubiquinone sequence. CoA, coenzyme A; LA, DHLA, Q<sub>red</sub>, Q<sub>ox</sub>.

Likewise, extracts from *Polygonum cuspidatum* containing *trans*-resveratrol and emodin were shown to protect the myocardium after ischemic events (112).

## 2. Neuronal Disorders

Neuronal disorders such as Parkinson's disease or Alzheimer's disease (113) gain increasing importance as a result of the increasing age of population.

Herbal extracts have been used as remedies for mental and general neuronal disorders historically and their use has dramatically revived in our days. Prominent examples are *Ginkgo biloba* extracts (114,115) and extracts from St. John's wort, *Hypericum perforatum* (116,117).

The goal, as in atherosclerosis and heart diseases, is to combine safe drugs (herbal extracts) with supplemented nutrition (novel foods, nutraceuticals, functional foods) in order to yield preventive protection. Two books addressing and perfectly summarizing these subjects should be mentioned in this context: the book comprising aspects of pharmacognosy by Bruneton (36) and that on functional food by Wildman (118). Both treatises discuss their respective fields exhaustively, not avoiding critical aspects.

In a 2001 review (119) another very important new field is addressed: "Medical molecular farming: production of antibodies, biopharmaceuticals, and edible vaccines in plants" open new visions and promise interesting future research areas. Recent developments of this rapidly growing research area of utmost commercial importance are critically discussed, with respect to environmental concerns. One of the authors principal points is that plant derived biopharmaceuticals are cheap to produce and store, easy to scale up for mass production, and safer than those derived from animals. That may be correct in many or most cases. There is almost nothing to add: this issue

is close to our own concern and research field. We therefore want to introduce our simplified scheme for testing plant activities in this respect.

## **B. Principles of Analyzing Reactive Oxygen Species and Testing for Antioxidative Capacities: Drug and Health Food Design**

Production of reactive oxygen species (ROS) by activated leukocytes or enzymes such as xanthine oxidase (XOD) plays an important role in the development of disease symptoms in animals. Destructive activities of ROS can be mimicked by biochemical model reactions simulating these disease processes and producing ROS, which in turn can be detected by derivatization of certain indicator molecules. Such in vitro model reactions thus contribute to our knowledge about potential dangers, increase the understanding of corresponding mechanisms, and analyze dose–response effects of drugs that act as antioxidants, food ingredients, or food additives (120) and analyze the effectiveness and quality of food processing (121,122). We compare polyphenols, coumarins, flavonoids, terpenoids, and complex plant extracts with well-known antioxidants that exhibit protective effects such as ubiquinol and vitamins (A, C, E.) or salicylic acid, thus allowing standardization of compounds or mixtures with unknown potential.

### **1. Biochemical Model Reactions for Testing Corresponding Potentials**

Biochemical model reactions, suitable for proving antioxidative and anti-inflammatory properties of the plant extracts mentioned comprise chemical reactions, enzyme-catalyzed reactions, and complex biochemical model reactions. The models generally consist of three functional units:

1. An activating reaction (light; reductant = “electron donor”)
2. An activatable molecule, which transfers the activation onto oxygen (redox cycle exciter pigment)
3. A detector (indicator) system, reacting sensitively with the individual ROS (KMB; ACC; unsaturated fatty acid; tyrosine), causing a specific signal (ethene; conjugated diene; nitrotyrosine).

### **2. Principle of Process Conduction**

In detail, the reactions based on the preceding principles were conducted as follows: the reaction mixtures containing the three components mentioned

were incubated for a specific period (30 or up to 60 min) in a reaction vessel at defined temperature (mostly 37°C). After (or during; see dienconjugation in LDL) the reaction, the derivatization of the indicator was measured. The reaction was conducted in the absence or presence of the test substances or drugs in order to study their influence on the reaction velocity. The reaction had to be controlled precisely so that no disturbances of the detection process or other false negative or false positive interactions occurred. Corresponding control reactions always had to be included.

In order to study the antioxidative properties of plant extracts three individual system categories required four different experimental setups, which are described in detail in the reports cited (for experimental details and medical background (see Refs. 49, 57, 120, and 123).

We generally used three categories of test systems:

Category I: Simple chemical systems

The Fenton/Haber–Weiss system

The dihydroxyfumaric acid–copper system

The Rose–Bengal system in light

The Peroxynitrite system ( $\text{ONOO}^-$ )

Category II: Enzymatic systems with

The xanthine oxidase reaction

The NAD(P)H-oxidase/diaphorase reaction

The lipoxygenase reaction

The myeloperoxidase reaction

Category III: Complex systems (57)

The myeloperoxidase-elastase/ $\alpha$ 1-antiprotease system

Low-density lipoprotein dienconjugation

Tyrosine nitration by peroxidase-nitrite or  $\text{ONOO}^-$

Activated neutrophil granulocytes in whole blood

These model reactions are taken as pharmacological proof in cases in which animal studies or epidemiological results give equivocal answers (see also: Ref. 124). The results allow indications as to the protective potential of the substances under discussion or of combinations of preparations as health-protecting additives in functional food.

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# 3

## Uptake and Transport of Xenobiotics

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### I. INTRODUCTION

#### A. Plant–Environment Relationships and Xenobiotics

Plants, like any other organisms, are able to exist as highly organized systems open to the environment. Plant life can only be maintained when energy and matter are taken up from the surrounding milieu, incorporated into the body, and eventually dissipated or released as heat or waste. Taking up substances from the environment and transporting them within the body are both characteristic traits and basic, indispensable prerequisites of plant life. But the flow of matter and energy between the environment and the interior must be under physiological control. Homoeostasis, the control of the internal milieu of the organism, is another indispensable property of life, which requires that the organism gain control over the quality and quantity of material exchange with the environment and over the temporal pattern of exchange.

The evolution of higher plants into the multicellular, photoautotrophic and land-dwelling organisms successfully adapting to almost any type of habitat found on Earth has produced an array of well-suited structures and mechanisms enabling plants to exchange substances with the environment while keeping their internal composition under control. In this way, plants are able to handle inorganic as well as organic substances, which may be in the gaseous, liquid, solid, or dissolved state.

The ways plants allow and/or control fluxes between the environment and the interior depend on the physical and chemical properties of the substances involved. Therefore, a priori, a plant cannot discriminate between beneficial and detrimental compounds or between those of biogenic

and anthropogenic origin. Toxic substances, in principle, behave in the same way as nutrients if both have comparable physicochemical properties. Toxicity, i.e., the effect of a poison on living systems, is a property without relevance to the ports of entry into and the mechanisms of transport within a plant. Thus, a toxicant is taken up into the plant and moved around within it according to the rules applying to whatever substance matches its profile of chemical and physical properties (1).

It is the objective of this chapter to outline general principles governing the uptake into and transport within a terrestrial higher plant. The emphasis is on the entry and dispersal of lipophilic organic compounds via the above-ground parts of plants because this type of xenobiotics plays major roles as environmental pollutants (2, 3) and as active ingredients of agrochemical formulations (4–6). The diversity of potential toxicants, however, is so large and detailed knowledge on uptake and transport at the same time so sparse that an adequate treatment has to be based on (semi)quantitative rules and an intuitive understanding of the processes and parameters involved rather than on direct experimental evidence specific for each potential contaminant. And finally, only the two most important components of toxicant entry into and movement within the plant are covered in this treatment: (a) the uptake of compounds freely available in the soil, the atmosphere, or (wet or dry) precipitation and (b) the long-distance transport within the plant. These considerations exclude processes such as the uptake from pesticide formulations and their neat residues remaining on plant surfaces as well as transport phenomena on intra- or intercellular scales.

## **B. Properties of Toxicants Relevant to Uptake and Movement in Plants**

The routes of uptake into the plant and their control by barriers as well as the mechanisms leading to the internal distribution favor some compounds while they discriminate against others. This characteristic leads to the selection of subsets of toxicants available to the plant out of the multitude of substances potentially present in the environment. The criteria acting during this selection are derived from two basic steps involved in any process of uptake and/or distribution: (a) the dissolution in an aqueous milieu and (b) the diffusive transport across membranes of lipoid nature. Any substance unable to dissolve in water and to move across lipid membranes in appreciable amounts does not reach its site of biological action within the plant cell.

This reasoning suggests that there should be specific combinations of physical and chemical properties of organic compounds in the environment that—via uptake and distribution—provide access to the sites of biological

(toxic) action within a plant. For solutes, the saturation concentration of the compound in water (aqueous solubility) is the most widely used parameter for describing the interaction of a substance with an aqueous medium (7). The tendency of a substance in aqueous solution to move into and to accumulate in an adjacent organic phase is expressed by the dimensionless partition coefficient. As a standard organic phase 1 octanol is employed such that the most widely used measure for the relative lipophilicity of a compound is the 1-octanol–water partition coefficient ( $K_{ow}$ ) according to

$$K_{ow} = \frac{C_o}{C_w} \quad (1)$$

where  $C_o$  and  $C_w$  are the equilibrium concentrations of the substance in the 1-octanol and water phases, respectively (8). Water solubilities and 1-octanol–water partition coefficients are inversely related (7, 9, 10). With weak organic acids or bases, aqueous solubility, the transport across lipid membranes, and the partitioning between aqueous compartments with differing pH strongly depend on the acidity constant  $pK_a$  (11).

For volatile or semivolatile toxicants the saturation vapor pressure ( $P_s$  in pascals) is an additional parameter determining the availability of a compound for uptake into and transport within plants (11). Values for the physicochemical properties can be either obtained from reports on experimental measurements or estimated from the chemical structure or from easily accessible fundamental properties of the compounds (12).

## II. UPTAKE FROM THE ENVIRONMENT

Terrestrial plants, in contrast to the majority of other organisms, simultaneously live in two major compartments of an ecosystem: the soil compartment (lithosphere) and the air compartment (atmosphere). Thus, the below-ground and the above-ground parts of plants experience physical, chemical, and biological environments that can hardly be more different. The differences have to be considered when the availability and uptake of toxicants from the respective compartments are to be assessed.

The soil environment is a primarily solid compartment with varying amounts of aqueous solutions and gaseous mixtures filling the pores between the organic and inorganic portions of the soil matrix. The below-ground parts of plants (roots, rhizomes) are primarily exposed to solutes in the bulk of soil water or associated by adsorptive and other mechanisms to soil



particulate matter. The gaseous phase of the soil is a reservoir for xenobiotics if they are sufficiently volatile, such as organic solvents or fumigants.

Xenobiotics originating from the atmosphere may be in varying physical states when they impinge on the above-ground parts of plants (leaves, stems, flowers, fruits). Obviously, toxicants may be present in the gas or vapor state, but—often much more important—they arrive at the plant surface as, or associated with, dry particulate matter (aerosols) or dissolved in rain or fog water (3, 13, 14). In comparison to conditions in the soil compartment, convection and mixing of the atmosphere and its content of toxicants are very rapid as a result of laminar and turbulent flows in the gas phase.

The parts of higher plants buried in the soil and reaching out into the atmospheric compartment, respectively, have widely differing functions in terms of uptake and transport and, consequently, show specific adaptations on the organ, tissue, and cell levels to their respective functions. In general, the root system is dedicated to the uptake of aqueous solutions and the above-ground parts of plants are mainly engaged in gas exchange. Stems and leaves are specifically protected against the loss (and uptake) of liquid water and many solutes by the cuticle (in the case of primary plant organs) and by a periderm (for secondary parts) (15).

### A. Root Uptake

The roots extract water and solutes from the soil and channel them into the long-distance transport system called *xylem* that leads to a distribution over the whole plant. Solute dissolved in soil water may reach the interior of the roots via two routes of entry: (a) the root hairs (delicate prolate protrusions from rhizodermal cells) (16) and (b) the hyphae of mycorrhizal fungi roots engaged in the mycorrhizal symbiosis (17), which generally are devoid of root hairs. In both cases, the plant root system develops enormously effective surface areas in contact with soil water and matrix. It has been reported, for instance, that a single plant of rye (*Secale cereale*) has approximately  $1.43 \times 10^{10}$  active root hairs making up a total surface area of approximately  $400 \text{ m}^2$  (18, 19). The total surface area of the extremely fine mycorrhizal hyphae extending from the root cortex into the surrounding soil cannot be measured exactly. However, it is assumed to even exceed that of the hairs of roots without mycorrhizal fungi.

The uptake of water and solutes is restricted to the very young and still elongating parts of the root system. An appreciable volume fraction within these fine roots is freely accessible to water and solutes. This volume fraction, called the *apparent free space* (AFS), contributes 8% to 25% to the total fine root volume. Structurally, the AFS mainly consists of the cell walls

of the root hairs and the root cortex cells and, therefore, is equivalent to the apoplast of the peripheral root layers. The uptake of solutes into the AFS proceeds by either passive diffusion or solvent drag; it is practically nonselective and reversible. In the case of diffusion, this movement is driven by a gradient of the chemical potential of the solute. With solvent drag, the solutes are carried along with a mass flow of water, and, in consequence, the driving agent for this process is the gradient of the water potential between the root interior and the soil water.

Among organic xenobiotics present in the soil, the mechanisms of entry into the root described apply only to those considerably soluble in water, i.e., polar nonelectrolytes or the dissociated forms of weak organic electrolytes. Apolar, more lipophilic compounds are not freely available in the soil water but instead are more or less firmly associated with clay particles and the organic carbon fraction (20, 21). So, the root uptake of highly apolar xenobiotics such as polychlorinated dioxins, which under certain circumstances may be present at considerable concentrations in soils, is practically negligible and above-ground plant contamination derives almost exclusively from dioxins volatilized from the soil surface (22–24).

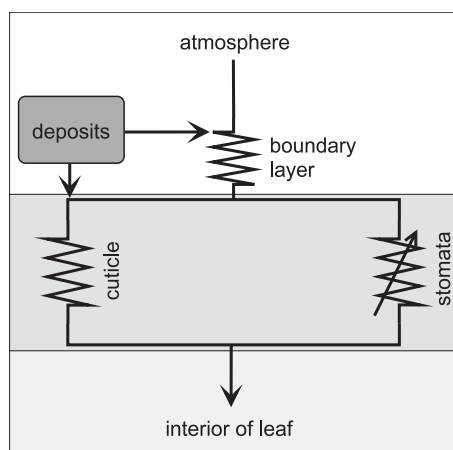
When polar or charged organic solutes have entered the root apoplast their further fate within the plant depends on their ability to reach the long-distance transport tissues located in the central cylinder of the root. In principle, for polar solutes, as for water, two pathways lead across the root cortex into the central stele: (a) an apoplastic path where the solutes diffuse exclusively in the cell-wall continuum and (b) a symplastic path where solutes are taken up into the cells across the plasmalemma and then stay in a symplastic continuum by moving from cell to cell via the plasmodesmata bridging the cell walls of adjacent cells (25, 26). Whereas the symplastic pathway is uninterrupted from the cortex into the central cylinder, the apoplastic diffusion of solutes encounters a barrier at the inner margin of the cortex. There, the radial cell walls are encrusted with a suberinlike material (27) that impedes the further movement of solutes. This cylindrical tissue, called the *endodermis*, is only one-cell-layer thick. Further progression to the central stele can be achieved only by solutes' circumventing the endodermal barrier by crossing the plasma membrane and continuing on the symplastic pathway. This is considered as providing the plant with at least some control of the influx of solutes from the soil solution (28–30).

## B. Uptake by Above-Ground Plant Organs

When comparing the basic principles of uptake into roots and leaves one may be surprised by a striking parallelism between the entry of solutes into the roots and the exchange of gases by leaves. In both cases, uptake and

release are essentially not inhibited by any permanent structural barrier, suggesting that the free flow of solutes and gases into the root cortex and the leaf mesophyll is evolutionarily advantageous. In contrast to roots, leaves possess a superficial barrier against the exchange of matter between the surrounding environment and the interior: leaves, primary stems, and fruits are covered by a low-permeability cuticle that, in most cases, is perforated by adjustable valves, the stomata. Thus, the free, concentration-gradient-dependent exchange of gases and vapors is possible only when the stomata are open. Stomatal opening and closure, in turn, are under strict physiological control, which depends on internal and external factors (31).

This picture applies to the naturally occurring polar gases and vapors such as  $\text{CO}_2$ ,  $\text{O}_2$ , and water, as well as to potentially toxic inorganic gases such as  $\text{SO}_2$ ,  $\text{O}_3$ , and  $\text{NO}_x$ . For lipophilic vapors and for all types of liquids, solutes, and solids the stomatal pathway is not passable or only negligibly so. For these compounds, the route of entry into the above-ground parts of higher plants is via the cuticle or the periderm. Thus, different pathways lead from the atmospheric environment into the leaf mesophyll, depending on the physical state and the chemical nature of the toxicant in question as well as on stomatal closure or opening in the case of gaseous polar compounds (Fig. 1).



**Figure 1** Network of resistances encountered by xenobiotics taken up into plants via the leaf-atmosphere interface. A substance deposited on the leaf surfaces or originating from the atmosphere in the gaseous state principally can enter the leaf via two parallel routes, the cuticle and the stomata. Which of the two pathways is preferred depends on the physicochemical properties of the chemical.

## 1. The Cuticular Pathway of Foliar Uptake

The cuticular pathway for uptake into or release from the leaf is the exclusive one for scarcely volatile to involatile solutes contained in liquid or solid deposits on the leaf surface. This class of compounds comprises all polar electrolytes and nonelectrolytes and apolar organics with low vapor pressures including a wide variety of organic xenobiotics in the environment and of active ingredients and adjuvants of pesticidal formulations. Compounds with higher volatility, in principle, can take two parallel paths to the interior of a leaf as long as the stomata are open (Fig. 1). The pathway preferred depends on the lipophilicity of the compound (i.e., its 1-octanol–water partition coefficient) and on its saturation vapor pressure (32–34). With stomata closed or absent (in the case of some fruits) the uptake of all volatiles and semivolatiles is restricted to the cuticular pathway.

Transport of material across the cuticle-covered plant–atmosphere interface arises from nonequilibrium conditions: the chemical potentials of solutes or vapors differ between the outside and the interior of the leaf. The important fact, however, is not that a xenobiotic is transported at all (at a dose that, from the point of view of toxicology, may be irrelevant) but the time required for biologically relevant amounts of material to be taken up. This is to say that the decisive function of the plant cuticle is not to act as an absolute barrier but to exert kinetic control over uptake (and loss).

The mechanism of transport across the plant cuticle is diffusion along a gradient of chemical potential. During this process, the cuticle behaves as a solution–diffusion membrane (35). This model of transport assumes that the movement of molecules across the cuticle follows a three-step process starting with the partitioning of a toxicant from the outside medium into the cuticle. In the second step, the substance moves across the cuticle by diffusion and in the third step leaves the cuticle at the other interface again by a partitioning process (36).

This overall process of transport across the cuticular pathway can be visualized as the diffusion of a compound from a donor compartment (e.g., deposits on the surface of the cuticle) to a receiver compartment (e.g., the interior of the leaf) with the compartments separated by the cuticle. Under steady-state conditions, the amount permeated linearly increases with time and the flow  $N$  is given by

$$N = PA(C_d - C_r) \quad (2)$$

where  $C_d$  and  $C_r$  are the concentrations in the (aqueous) donor and receiver compartments, respectively;  $A$  is the area of the cuticle exposed; and  $P$  is the

permeance of the cuticle. At low concentrations and with nonelectrolytes, the gradient of chemical potential can be substituted by the difference of concentrations as driving force. In the steady state  $C_d = \text{constant}$  and  $C_d \gg C_r$ , and hence Eq. (2) can be simplified to

$$N = PAC_d \quad (3)$$

The permeance  $P$  is the proportionality coefficient relating the flow to the area exposed and the driving force. For homogeneous membranes and aqueous donor and receiver compartments, the permeance is directly related to the fundamental transport-related properties of the membrane by

$$P = \frac{K_{cw}D}{\Delta x} \quad (4)$$

where  $K_{cw}$ ,  $D$ , and  $\Delta x$  are the cuticle–water partition coefficient [see Eq. (1)], the diffusion coefficient of the xenobiotic in the membrane, and the thickness of the membrane, respectively. Even though the assumption of homogeneity is not valid for the plant cuticle (37), Eq. (4) is a useful tool for analyzing and understanding cuticular permeability in terms of fundamental properties. Qualitatively, Eq. (4) shows that permeance, and thus flow across a cuticle, increases both with increasing relative solubility and with mobility of the permeant in the cuticle.

Applying this formalism, the transport of nonelectrolytes along the cuticular pathway has been analyzed on different levels of complexity and integration by studying intact leaves, isolated cuticular membranes, and reconstituted films of cuticular waxes.

## 2. Experimental Determination of Transport Properties

Cuticular transport into intact leaves can be examined by measuring foliar uptake of molecules applied as aqueous solutions to the leaf surface (38, 39). The experiments must be performed in the steady state, allowing only a negligible fraction of the total amount of substance applied to be taken up during their course. Under such conditions, permeances can be determined.

Obviously, use of intact leaves for investigating the cuticular pathway of foliar uptake is the system closest to biological reality. However, it is able to provide only a very limited insight into the fundamental mechanisms of cuticular transport. This limitation is due to the difficulty of controlling all factors influencing the flow as strictly as necessary. As a consequence,

enzymatically isolated cuticular membranes have been preferred for mechanistic studies and have proved to be powerful tools for analyzing cuticular penetration (6, 40–42).

One of the major advantages of using isolated cuticles is the opportunity to investigate the effects the different constituents of cuticular membranes exert on transport properties. Operationally, the cuticle of plants can be considered as a multicomponent system consisting of at least three major fractions: (a) the insoluble cutin polymer, (b) the soluble cuticular waxes, and (c) polar components (polysaccharides, phenolics) (43–45). An important question is, To what extent do waxes and cutin each contribute to the barrier properties of plant cuticles?

This question can be answered experimentally because, with isolated cuticles, the permeances of both the intact cuticular membranes (CMs) and of polymer matrix membranes (MX; with cuticular waxes removed by organic solvent) can be determined. The increase of permeance after removal of the cuticular waxes, expressed by  $P(\text{MX})/P(\text{CM})$ , is a measure for the contribution of the waxes to the total barrier of the cuticle. With leaf cuticles from *Citrus aurantium* L. the removal of waxes increased permeance by factors from 10 to 1300, depending on the nature of the permeant. This means that cuticular waxes are responsible for 90% to 99.9% of the total resistance of cuticular membranes. Similar results have been obtained with other species and with cuticles from other organs. This finding clearly identifies the cuticular waxes as the principal barrier controlling the movement of molecules across plant cuticles.

The recognition of the cuticular waxes as the transport-limiting barrier of plant cuticles leads to a further question: Are the properties of this barrier distributed evenly across the thickness of the cuticle (from less than 1  $\mu\text{m}$  to more than 10  $\mu\text{m}$ ), or is the cuticle an asymmetrical membrane with respect to diffusive transport? Experimental evidence has shown that cuticular membranes are indeed highly asymmetric in terms of transport properties (46). The barrier consisting of cuticular waxes is located at or close to the anatomically outer surface of the cuticle. A large part of the cuticle situated adjacent to the inner surface has negligible barrier properties and probably represents the polymer matrix devoid of waxes.

On the basis of this functional (rather than fine-structural) evidence (46, 47) a tentative model of the plant cuticle has been proposed. According to this model, the cuticle consists of an “inner volume element” including the cuticular pegs, an “outer volume element” or “skin,” and a more or less rough cover of epicuticular waxes. The “skin” embodies the transport barrier. At the moment, it is unclear whether this skin is a very thin layer of wax-impregnated cutin or a continuous film of epicuticular wax covering the outer surface of the cutin matrix.

This asymmetrical nature of the plant cuticle has been the basis for a further type of experiment that provides an improved understanding of diffusion in the actual transport barrier. In this experimental setup, an isolated cuticular membrane is again loaded with a test compound that; subsequently, is desorbed across the outer surface of the cuticle by a suitable desorption medium (48, 49). Diffusional transport across the cuticular barrier ("skin") differs from the condition described by Eq. (3) in two aspects: (a) the driving force is the concentration difference between the cuticle and the aqueous receiver phase (and not that between two aqueous phases), and (b) the driving force decays with time. The change of concentration in the cuticle (i.e., donor) compartment is given by

$$\ln \frac{C_c^t}{C_c^0} = - \frac{P^* \cdot A \cdot t}{V_c} \quad (5)$$

where  $C_c^t$  and  $C_c^0$  are the concentrations in the cuticle at time  $t$  and at time 0, respectively;  $P^*$  is the permeance based on the concentration in the cuticle as the driving force; and  $A$  and  $V_c$  are the area and volume of the cuticular membrane exposed, respectively.

Experimentally, the ratio on the left side of Eq. (5) is determined according to

$$\frac{C_c^t}{C_c^0} = 1 - \frac{M^t}{M^0} \quad (6)$$

with  $M^t/M^0$  the ratio of the amount of the substance desorbed from the cuticle up to time  $t$  and the total amount in the system. Combining Eqs. (5) and (6) and solving for  $P^*$ , the relationship

$$P^* = - \frac{\ln(1 - M^t/M^0)}{t} \cdot \frac{V_c}{A} = k^* \cdot \frac{V_c}{A} \quad (7)$$

is obtained. The rate constant  $k^*$  of the desorption process is given by the slope of a plot of  $\ln(1 - M^t/M^0)$  vs.  $t$ . Permeances according to Eq. (3) are related to  $P^*$  by

$$P = K \cdot P^* \quad (8)$$

Rate constants  $k^*$  and  $P^*$  are directly proportional to diffusion coefficients. They describe exclusively the mobility of a compound in the rate-limiting barrier of the cuticular membrane and do not contain any contribution from

relative solubility. This special property made rate constants a powerful tool for analyzing the dependence of the mobility of a permeant in the barrier on the nature of the compound, the species or organ of plant studied, and the effect of other factors (50–54).

One of the major facts that became clear from studies using isolated cuticular membranes was the pronounced variability of permeances between plant species and between cuticles from different organs. For example, permeances for 2,4-dichlorophenoxyacetic acid (2,4-D) varied in a set of cuticles from 11 species over a range from  $1 \times 10^{-10}$  (*Ficus elastica* leaf) to  $2.7 \times 10^{-8} \text{ m}\cdot\text{s}^{-1}$  (*Capsicum annuum* fruit). The permeabilities of fruit cuticles were consistently higher than those of cuticles isolated from leaves. A major part of these differences appear to be due to differences in the mobility of the permeant in the transport-limiting barrier. Rate constants from desorption experiments were up to a factor of 10 higher with *Capsicum annuum* fruit than with *Citrus aurantium* leaf cuticles. Similar results were obtained when the permeability to water was studied (55–57).

### 3. Modeling Cuticular Uptake

The single two most important parameters determining the interaction of lipophilic toxicants with the plant surface and the subsequent uptake into the plant interior are the cuticle–water partition coefficient and the cuticular permeance. As outlined, direct experimental measurement is feasible only for a small fraction of the large number of environmental xenobiotics. Thus, methods for estimating these parameters from easily accessible properties of the chemicals have been developed.

For the prediction of the cuticle–water partition coefficient  $K_{cw}$ , different approaches have been used so far, relying either on fundamental properties or on molecular structure. A quantitative property–property relationship (QPPR) was established between the cuticle–water partition coefficient and the 1-octanol–water partition coefficient (42)

$$\log K_{cw} = 0.057 + 0.970 \log K_{ow} \quad (r = 0.987) \quad (9)$$

A similar correlation, but with a slightly lower degree of determination, was obtained between  $K_{cw}$  and the aqueous solubility of the compound in water ( $S_w$  in  $\text{mol l}^{-1}$  at  $25^\circ\text{C}$ ) (42):

$$\log K_{cw} = 1.118 + 0.569 \log S_w \quad (r = 0.978) \quad (10)$$

The flow of organic nonelectrolytes across isolated plant cuticles can be measured experimentally and the permeance  $P$  derived according to Eq. (3).



This has been done for a number of chemicals of diverse structures and physicochemical properties (41, 58–60). The values for cuticular permeances obtained so far range over four and a half orders of magnitude from  $1.43 \times 10^{-11}$  for 5,6-dimethyl-2-dimethylamino-pyrimidin-4-yl-N,N,-dimethyl-carobonate (Primicarb) to  $8.60 \times 10^{-7} \text{ ms}^{-1}$  for hexachlorobenzene. A comparison with the respective cuticle–water partition coefficients shows that permeances are strongly influenced by the partition coefficient term in Eq. (4).

There is also considerable variation among cuticular permeances determined for one chemical and the cuticles of a number of plant species. A study with 2,4-D and the cuticles from 11 plant species showed that permeances ranged over two orders of magnitude (59). The cuticles of sour orange (*Citrus aurantium*) were at the lower limit of the range; those of fruits of garden pepper (*Capsicum annuum*) formed the upper limit. Thus, permeances can be assumed to vary by a factor of 100 among species.

The observation that cuticular permeances were correlated to cuticle–water partition coefficients again led to the attempt to establish quantitative relationships for predicting permeances of *C. aurantium* leaf cuticles from more fundamental and readily accessible parameters. Such correlations have been successfully established between the cuticular permeance and the 1-octanol–water partition coefficient

$$\log P = 0.704 \log K_{ow} - 11.2 \quad (r = 0.91) \quad (11)$$

and between  $P$  and the cuticle–water partition coefficient

$$\log P = 0.734 \log K_{cw} - 11.3 \quad (r = 0.95) \quad (12)$$

Using the cuticle–water instead of the 1-octanol–water partition coefficient as predictor somewhat increases the predictive power of the relationship. It should be stressed in this context that Eqs. (11) and (12) have been derived for *C. aurantium* leaf cuticles and may give incorrect results for different types of cuticles. Attention should again be paid to the fact that permeances may vary between different plant species by up to two orders of magnitude.

#### 4. Cuticular and Stomatal Uptake of (Semi)Volatile Xenobiotics

Some organic xenobiotics reaching the plant from the atmosphere may have a sufficient vapor pressure and thus volatility at ambient temperatures that, when stomata are open, both the cuticular and the stomatal pathway may be passable. Both pathways are in parallel such that individual conductances add up to the conductance of the leaf surface (Fig. 1). Total conductances for the atmosphere-to-leaf transfer of the xenobiotics have been estimated

from experimentally determined cuticular permeances and the properties of a representative leaf. A further assumption was that the stomata of the leaf were, on the average, open during half of the period of exposure.

The cuticular component of the total flow of a chemical in the vapor phase from the turbulent atmosphere into a model leaf can thus be estimated and its dependence on fundamental physicochemical properties of the xenobiotic be explored systematically. It can be predicted that compounds with a high volatility (expressed as high air–water partition coefficients) enter the leaf exclusively via the stomata. Within the range of realistic values of 1-octanol–water partition coefficients, lipophilicity does not have an effect. When decreased volatility is assumed, the cuticular pathway is predicted to become increasingly important. More than 50% of the total flux of compounds having low volatility (logarithms of air–water partition coefficients of  $-3$ ,  $-5$ , or  $-7$ ) can be estimated to enter the leaf via the cuticle when their log 1-octanol–water partition coefficients were assumed to be approximately 6.7, 4, 1, respectively.

This clearly emphasizes the importance of cuticular penetration for the uptake of the large number of organic pollutants that have low vapor pressures. In many cases, the physicochemical properties of the compounds and the transport properties of leaf surfaces keep open only one port of entry into plants, namely, the diffusion across the cuticle.

### III. TRANSPORT WITHIN THE PLANT

The division of labor among cellular compartments, cells, tissues, and organs characteristic for plants as multicellular and highly differentiated organisms requires a multitude of transport processes over varying distances. As described for the uptake into the plant across its boundaries to the environment, internal movement is again (at first approximation) independent of the toxic properties of the xenobiotic but is massively influenced by the physicochemical properties of the compounds in question. Toxicity affects translocation only if the toxicant inhibits cellular mechanisms of active, or enzymatically facilitated transport.

The mobility of a toxicant may be altered within the plant by physicochemical processes; pH-dependent dissociation and protonation are the most important ones. Metabolism may also interfere with the xenobiotics by chemical modifications such as conjugation or oxygenation. Thus, the mobility of the actual xenobiotic species or metabolite present within the plant may be different from that of the original toxicant and, therefore, may exhibit improved or impeded rates of translocation.

Long-distance transport of toxicants taken up by either the roots or the above-ground parts of higher plants can, in principle, take place in two vascular systems connecting all major parts of higher plants, the xylem and the phloem.

### **A. Xylem Transport**

The xylem is the long-distance transport system of higher terrestrial plants for water and small polar solutes of both inorganic and organic origin. The solutes are dragged along with a continuous flow of water from regions of high water potential to those of low water potential. In most cases this means transport from the root tips through the stem to the sites of water consumption in the shoot. Water is consumed by a plant mostly by two processes: transpiration by the above-ground parts and growth of new tissue. Therefore, the material dissolved in the xylem content eventually turns up and, in the case of transpiring organs, accumulates at these sites. Within a leaf, such substances tend to accumulate around the leaf margin and toward the leaf tip.

Predominant movement of a given toxicant in the xylem does not mean that the substance remains in the apoplast once it has reached the leaf via the transpiration stream. The substance may redistribute between the symplast and the apoplast according to the specific conditions (especially the pH) in the different compartments of the leaf tissue (61).

Having outlined the apoplastic route for xenobiotic translocation in the plant we may now attempt to specify the properties a toxicant must have if it is to be translocated effectively via the xylem. The prerequisites are that it must be capable of entering the apparent free space of the root cortex, passing through the barriers in the endodermis. Then it is dragged along with the water flowing upward in the xylem, depending on the gradient of water potential between the root and the atmosphere surrounding the above-ground parts of the plant. As these processes primarily involve aqueous solutions, the first requirement is that the substance be readily soluble in water.

In addition, the substance must be sufficiently hydrophilic to avoid partitioning into the lipid materials that it may encounter along its path. However, any solute taken up by the root must also have sufficient lipophilic properties to pass through the barriers of the endodermis or to circumvent them by partitioning into the symplastic pathway. Thus, the properties required for apoplastic long-distance transport appear somewhat contradictory. In practice, however, it appears that at least some mobility in the apoplast is found over a wide range of different polarities (62).

As outlined, protonation and dissociation of weak electrolytes may contribute to the actual polarity of the predominant species of a substance under the specific conditions within a tissue or a cell. During apoplastic transport, ion exchange with components of the cell wall may severely interfere with and restrain the movement of the charged species of organic toxicants. As the net charge of the fixed ions in the apoplast is negative, cationic toxicants have a low mobility and are retained by the negative charges of the cell walls. This characteristic suggests an analogy between the apoplastic transport system for xenobiotics in plants and a chromatographic column in which the flow of water carries dissolved material with it that may partition into lipoid stationary phases or be retarded by ionic interactions (62).

## **B. Phloem Transport**

The mechanism of transport of both endogenous compounds and toxicants in the phloem has been, and still is, a subject of discussion. In any case, substances move from source areas, where they are synthesized (e.g., the leaves) or released at storage sites (e.g., the stems and roots), to sink areas, where they are metabolized or used otherwise. Solutes (primarily assimilates) are “pumped” actively (adenosine triphosphate [ATP] consumption) from a source into the sieve tubes of the phloem. This inflow of solutes causes a decrease of the water potential within the sieve elements in the region of the source, which results in an influx of water. In the sink region, solutes are actively exported from the phloem and water follows osmotically out of the sieve tubes. The mechanism of phloem translocation as proposed by the mass-flow hypothesis thus involves active transport at each end of the sieve tube, with the passive movement of assimilates and other solutes through the tube by means of osmotic pressure buildup and resultant bulk flow.

As discussed earlier, any compound (except water) taken up by the roots must cross the plasmalemma from the apoplast to the symplast in order to overcome the endodermal barrier. Thus, all substances that reach the central stele of the root are, at least in principle, able to cross cell membranes and, thus, should also be capable of entering the phloem. This consideration suggests that although, many compounds are capable of entering the phloem, only those compounds that, in addition, are retained in the sieve tubes are well translocated. Those moving freely forth and back across the plasma membrane of the sieve tubes preferentially are transported by the xylem because of the much greater volume flow in the xylem elements than in the phloem (63).

The translocation of a xenobiotic in the phloem consequently depends on two processes: (a) its uptake by the sieve tubes from the surrounding

tissues and (b) the retention of the compound in the phloem during translocation. Nonionized chemicals of intermediate membrane permeability are expected to show some phloem movement because they can enter the sieve tubes when local concentrations are high. Very polar nonelectrolytes do not permeate across the sieve tube plasmalemma and thus are not translocated in the phloem. More lipophilic compounds ( $\log K_{ow} = 1$  to 3) may cross the membranes easily and thus enter the phloem but immediately diffuse back into the much greater volume of the xylem and are translocated there.

It has been recognized that most of the phloem-mobile xenobiotics are weak electrolytes, mostly weak acids. Experimental and modeling approaches have provided sound evidence for the assumption that such compounds are phloem-mobile mainly because of iontrapping in the more basic phloem ( $\text{pH} \approx 8$ ) relative to the apoplast and a  $\text{pH}$  around 5.5 of the xylem. The optimal physicochemical properties of an organic xenobiotic for penetrating the plasmalemma surrounding the sieve tubes have a low degree of dissociation at the given  $\text{pH}$  (i.e., high  $\text{p}K_a$ ) and, for the nondissociated species, a 1-octanol–water partition coefficient in the range of 100. Although these properties ensure good uptake into the phloem they also lead to a rapid loss from the phloem. In order to be retained in the phloem and to be transported effectively within it, a weak organic acid must have a high degree of dissociation (i.e., low  $\text{p}K_a$ ) and the small fraction of nondissociated molecules present should be very polar ( $\log K_{ow} < 1$ ). Thus, the requirements for uptake and retention are in conflict, and, therefore, the optimal values for efficient phloem transport of weak acids must be a compromise between the requirements for the two contributing processes (63–68).

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# 4

## Air Pollution: Trace Gases as Inducers of Plant Damage

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### I. INTRODUCTION

The apparent local and global increase of certain atmospheric trace gases has become a worldwide focus of discussion since all living organisms and ecosystems are affected by these dry and wet emissions and subjected together to atmospheric and stratospheric “global changes” (1). During the last 20 to 30 years we had to learn that organic gaseous emissions such as methane (stemming from ruminants) together with other trace gases in the air (sulfur dioxide, different oxides of nitrogen, resulting from combustion or rhizospheral bacteria) undergo extremely complex reaction chains, partially dependent on light as driving force, and produce a plethora of new compounds that may either be toxic to animals and plants or have climate effects, or do both. Numerous symposia were held on this subject since the beginning of the awareness of the arising problems and a wealth of books have been published since then (2–11). In most of these reactions, oxidative processes and peroxides play a pivotal role, either as intermediates or as end products. This role of oxidative processes in air pollution had already been reported in the 19th century, when the production of acid rain was recognized as an oxidation by atmospheric hydrogen peroxide of sulfur dioxide stemming from coal combustion (12). Similarly, chlorophyll bleaching was described by the end of the 19th century as a process

catalyzed by oxidizing enzymes. Nevertheless we were greatly surprised by the overwhelming number of recent findings that oxygen toxicity is a dominant factor in general toxicological and pathological conditions.

II. GENERAL ASPECT OF THE BIOLOGICAL CHARACTERISTICS OF AIR POLLUTANTS

A. The Most Important Trace Gases

SO<sub>2</sub>, NO<sub>x</sub>, ozone, peroxyacetyl nitrate (PAN), ethene, and hydrogen fluoride (HF) and certain terpenoids (allelopathic factors) represent the most important trace gases in our atmosphere that act as plant toxins and may therefore be addressed as air pollutants with effects on plant growth and development (13,14). These trace gases stem from natural as well as anthropogenic sources. Approximative emission rates are summarized in Table 1.

B. Basic Types of Air Pollution

As shown in Table 2 six different types of trace gases and particles in the air may contribute more or less to air pollution with impacts on plants with regionally extreme differences in constitution and partition.

In Table 3 an overview of the most important physiological and ecological effects of relevant trace gases is presented.

**Table 1** Yearly Global Emission Rates (Approximations) of Important Air Pollutants

Compound	Emission rate, 10 <sup>9</sup> kg a <sup>-1</sup>			Main sources
CO <sub>2</sub>	830,000	na	800,000	Respiration, biological degradation
		a	30,000	Combustion
CO	3,400	a	—	Mainly combustion
Hydrocarbons (without CH <sub>4</sub> )	1,000	na	930	Trees
		a	70	Industry, motor vehicles
CH <sub>4</sub>	500	na	300	Swamps, rice fields
		a	200	Ruminants
SO <sub>2</sub>	400	na	20	Volcanoes
		a	380	Coal and oil combustion
NO <sub>x</sub>	160	na	10	Lightning
		a	150	Combustion

<sup>a</sup>a, Anthropogenic; na, nonanthropogenic.

**Table 2** Plant Damage: Origin and Types of Airborne Substances

Oxidative smog	NO <sub>2</sub> , peroxyacetyl nitrate, O <sub>3</sub> (Los Angeles type)
Reductive smog	SO <sub>2</sub> (London type)
Acids	Hydrogen fluoride, HCl, H <sub>2</sub> SO <sub>3</sub> , H <sub>2</sub> SO <sub>4</sub>
Dusts	Cement, sand, earth
Phytoeffectors	Ethene, herbicides, other plant protectants
Allelopathic factors	Terpenoids (e.g., monoterpenes)

**Table 3** Physiological and Ecological Effects of Relevant Air Pollutants on Humans and Plants: An Overview

NO <sub>2</sub>	Affects respiratory parameters; acts as plant fertilizer
O <sub>3</sub>	Influences membrane equilibria (transport blocker); affects respiratory parameters; may reduce resistance against viral infections; may reduce plant growth
SO <sub>2</sub>	Irritates eyes and airways; necrotizes plant tissues; acts as monocausal agent of forest die back ( <i>Waldsterben</i> )
Soot particles	Mutagenic, carcinogenic, and allergizing
SO <sub>2</sub> + soot particles	Produces acute toxicity at high concentrations; provokes leukocytes (alveolar macrophages) but impairs their bactericidal properties; potentially induces chronic respiratory diseases

Most air pollutants are reactive, small molecules and interact with biological molecules as a result of either their radical character (nitric oxides), strongly oxidative properties (ozone, PAN), or the reducing and acid-forming activities (nitric oxides; sulfur dioxide). An overview of the fundamental biochemical reactivities is given in Table 4.

More details on the biochemical background on reactions, physiological goals, and products are presented in Table 5.

## 1. London-Type and Los Angeles-Type Air Pollution

The term *smog* is derived from the contraction of the words *smoke* and *fog*; it stems from the early days of industrialization, mainly in England—and there specifically in London. This term has been (incorrectly!) transferred to other types of air pollution such as the Californian type.

As summarized in Table 6 the specific atmospheric conditions of the cities of London and Los Angeles represent two principles of major types

**Table 4** Biochemical Reactions of Potentially Toxic Trace Gases

CO	Binds to heme groups, thus reversibly blocking respiration; only a problem in high concentrations; ("garage effect," suicides)
NO	Acts as free radical; binds to heme groups; is rapidly oxidized to NO <sub>2</sub> ; is physiologically known as a regulator of blood vessel tonus
NO <sub>2</sub>	Acts as free radical, forming nitro compounds and peroxides; acts as precursor of strong acids; is physiologically known but of potential toxicity
O <sub>3</sub>	Acts as strong oxidant, forming peroxides; splits double bonds and oxidizes amino acids in proteins; is toxic (Los Angeles-type smog indicator)
SO <sub>2</sub>	Acts as precursor of strong acids; forms free radicals and rapidly activates peroxides, producing extremely reactive alkoxy or hydroxyl radicals (London-type smog indicator)
Soot particles	Contains aromatic hydrocarbons, nitroaromatics, quinines, and chelated transition metals, thus initiating toxic redox cycling; catalyzes autoxidation of SH groups and ascorbate; oxidizes SO <sub>2</sub> and NO <sub>2</sub> , yielding sulfuric and nitric acids (London-type smog indicator)

of air pollution with dramatic impacts on health of plants, animals, and humans. The main characteristics governing these two types are high air humidity, low temperature, and high sulfur dioxide in London and high temperature, high light intensities, and high volatile organic carbon (VOC) level in Los Angeles (15).

## 2. Formation of Photosmog ("Los Angeles" smog)

Photosmog was first recognized in the Los Angeles area in the 1940s. The geographical situation of the Los Angeles basin is particularly favorable for temperature inversions and therefore stable airmasses. In addition, the exhausts of the intensive motor vehicle traffic in this area are responsible for the accumulation of primary pollutants. Oxy radicals are of primary importance for the production of photochemical smog (15). Simulated photoreactions have been performed to show that reactions between NO<sup>•</sup> and oxygen seem to proceed via the catalysis of hydrocarbons (RH).

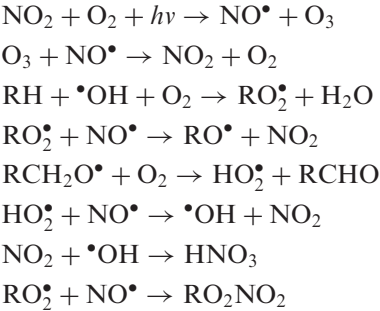
**Table 5** Examples of Experimental Phytotoxic Effects of Trace Gases and Aerosols

Trace gas aerosols	Reaction partner	Main reaction	Physiological reaction partner	Physiological reaction product
SO <sub>2</sub>		Competes with CO <sub>2</sub> inhibition	Carboxylases	Radicals
HSO <sub>3</sub> <sup>-</sup>	Probably thiols	?	Thioredoxine regulation system	
	Peroxides	Homolytical splitting	Lipid hydroperoxide	Different radicals, ethane
	Aldehydes	Addition	Pyridoxal phosphate	Addition products (inactivated vitamin)
NO <sub>2</sub>	Alkenes	Addition	Unsaturated lipids	Peroxides, nitrations
HNO <sub>2</sub>	Amines	Nitrosylation	Amines, amino acids	Alcohols, carboacids
O <sub>3</sub>	Alkenes thiols	Addition oxidation	Unsaturated lipids, cysteine, glutathione, protein	Aldehydes, ketones, cysteine, cysteine-sulfonic acid
Peroxyacetyl nitrate	Thiols	Oxidation	Methionine, cysteine	Methionine-sulfoxide
	Primary amines	Acetylation	Amines, amino acids	O <sub>2</sub> , HNO <sub>2</sub> , acyl amino compounds
	Aryls	H abstraction and reaction	Indole acetic acid (growth hormone) NO <sub>3</sub> <sup>-</sup> and O <sub>2</sub>	3-Hydroxymethoxyindole (growth inhibitor)

**Table 6** Comparison of London-type and Los Angeles-type Smog

London	Los Angeles
Peaks early in morning	Peaks midday
Temperature 30°–40°F	Temperature 75°–90°F
High relative humidity and fog	Low relative humidity and clear sky
Radiative or surface inversion	Subsidence or overhead inversion
Reducing atmosphere	Oxidizing atmosphere
Bronchial irritation	Eye irritation

The following equations (Scheme 1) represent the main chemical reactions of photosmog (see also the Chapter 2 on plant stress and oxy radicals):



**Scheme 1** Main chemical reactions of photosmog.

It has been mentioned that methane in rural areas may contribute significantly to the propagation of smog and radical formation in the atmosphere according to Scheme 2.

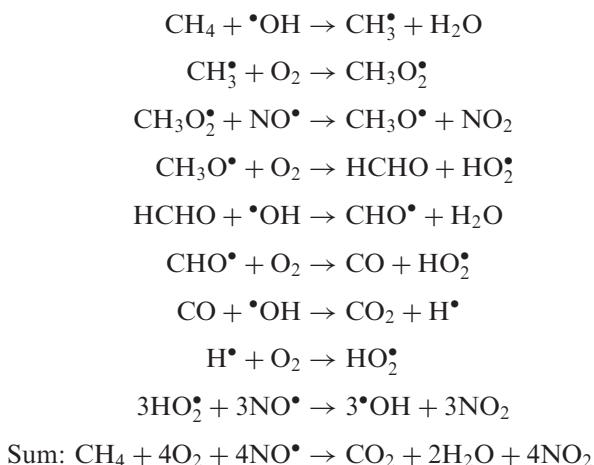
By this sequence,  $\bullet\text{OH}$  radicals are converted into  $\text{HO}_2^\bullet$  radicals, which regenerate  $\bullet\text{OH}$  radicals in the presence of  $\text{NO}^\bullet$ . At low  $\text{NO}^\bullet$  concentrations chain-breaking reactions predominate.

**C. Criteria for Evaluation of Air Pollutants and Their Effects**

**1. Concentrations and Exposures**

Impurities of clean air mainly occur in the range of parts per million (ppm) and parts per billion (ppb):

$$\begin{aligned} \text{ppm} &= \text{parts per million; dilution in air: } 1 : 10^6 \\ \text{ppb} &= \text{parts per billion; dilution in air: } 1 : 10^9 \end{aligned}$$



**Scheme 2** Photo-oxidation of methane.

These mixing ratios in air may be converted into mass concentrations (milligrams per cubic meter [ $\text{mg}/\text{m}^3$ ]; micrograms per cubic meter [ $\mu\text{g}/\text{m}^3$ ]) according to the formula  $[\text{ppm}] \times \text{molecular weight/mole volume} = [\text{mg}/\text{m}^3]$  (under  $0^\circ\text{C}$  and 1013 mbar). Exposition times are mostly given as short time exposures (30–120 min) or long time exposures. Short-time exposures at high concentrations mainly yield acute, visible symptoms; long time exposures at medium or low (i.e., environmentally realistic) concentrations yield chronic symptoms without distinct effects. Short time exposures with high concentrations are often used for studying biochemical reaction mechanisms and follow-up reactions. Long time exposures are useful for providing tools for administrative and thus political and governmental measures.

## 2. Determination and Quantification of Plant Injury and Damage

The terms *injury* and *damage* represent different views of impacts of air pollutants.

Whereas *injury* describes the impacts, visible or directly measurable on the plant (for example, percentage destruction of leaf area), *damage* means the loss of harvestable or marketable plant material. To give just one example: We compare the damage caused at identical injury, for example, 30% necrotic leaf area, of radish and lettuce leaves: since radish on the market is only estimated as value and intactness of the root (in the preceding case 0% damage), lettuce with 30% necrotic leaves may completely ( $\gg 80\%$  damage) lose its marketability.



*Approximation of yield losses* may be achieved by the following regression line:

$$y = a + bx$$

where

- $y$  = yield percentage of an unexposed control
- $a$  = constant number of ca. 100%; experimentally determined
- $b$  = descendant (slope) of the linear regression of the yield–destruction values; experimentally determined
- $x$  = percentage of the leaf surface destroyed (necrotized) or removed

For example, Alfalfa (*Medicago sativa*) was treated with 1–5 ppm SO<sub>2</sub> for 1–2 h and the following dependencies on the number of treatments have been obtained:

Number of treatments	Regression line
One treatment	$y = 99.5 - 0.30x$
Two treatments	$y = 95.5 - 0.49x$
Three treatments	$y = 96.5 - 0.75x$

From this experiment it becomes clear that the absolute value of the slope of the yield–leaf destruction curve increases with the number of treatments. The results of other cultures differ from those of alfalfa, since alfalfa is a fodder and the yield is thus directly dependent on the mass produced. For barley, wheat, or cotton air pollution has a different influence at the onset of flowering or fruit development, as shown for SO<sub>2</sub> treatment of barley:

- Treatment during the early vegetative state:  $y = 98 - 0.06x$
- Treatment during onset of flowering:  $y = 98 - 0.4x$

Table 7 explains that yield losses depend on the following:

1. The individual culture and the relevant plant part or organ (radish, beans)
2. The exposure periods and the intervals between exposure and harvest (grape wine)
3. The individual threshold values of the culture (spinach).

**Table 7** Crop Yield Losses of Different Cultures after Ozone Exposure

Plant	Experiment	Concentration and time	Effect (% loss when compared to control) <sup>a</sup>	
			Loss, %	Organ
Radish	Greenhouse	2–140 µg/m <sup>3</sup> ,	54	Root (FW)
		5 wks, 5 d/wk	20	Leaf (FW)
Bean	Greenhouse	300 µg/m <sup>3</sup> , 2 h/d	33	Whole plant
		63 d	46	Whole fruit
Spinach	Open field	200 µg/m <sup>3</sup> , 7 h/d	32	Leaf (FW)
		37 d		
		280 g/m <sup>3</sup> , 7 h/d	72	Leaf (FW)
Grape wine	Open field	37 d		
		Many times, 50 µg/m <sup>3</sup> (May–Sept.)	12 61	1-Year Grapes 2-Year Grapes

<sup>a</sup>FW, Fresh weight.

A further complication in the estimation of damage arises from the availability of the air pollutant, i.e., the experimental design during evaluation of the indicated parameters.

In most cases the plants are exposed to the trace gas in closed chambers or the air–trace gas mixtures are blown through (across) the exposure vessels. When the activity of ribulose biphosphate carboxylase oxygenase (RUBISCO) was examined in spinach leaves the degree of damage ( $S$ ) was not simply related to the product of time of exposition ( $t$ ) and the concentration of the trace gas ( $C$ ) according to

$$S = C \times t$$

The velocity of the gas stream is also of major importance. Therefore the preceding equation has to be adapted:

$$S = C_a \times t$$

where  $C_a$  is the product of SO<sub>2</sub> concentration and velocity (milliliters per minute [ml/min]) of the gas stream in the chamber. Overall it becomes evident that the quantification of damage of a culture by air pollutants is not a simple straightforward application of the dose–effect equation but depends on a plethora of special circumstances during the exposure and individual plant characteristics such as history, i.e., pretreatments or exposures. Many of these existing data related to environmental toxicology have been summarized in reviews and books cited previously (2–11).

### III. GENERAL BIOCHEMICAL ASPECTS OF REACTIONS OF AIR POLLUTANTS

#### A. Macroscopic, Cellular, and Subcellular Effects

##### 1. Visible Damage

Visible indications of impacts of air pollutants on plants are disturbances of growth and development such as stunting, cankers, epinastic leaf deformations, chloroses, and necroses. An early sign often is water logging in leaves, which later develops into zones of brown, dead tissue (necroses). Acute damage is mostly visible as mesophyll damage with intact phloem and xylem vessels. These macroscopic symptoms usually indicate acute exposure to above-threshold toxin levels.

The following symptoms may occur:

At low, acute levels of

1. PAN, HCl, Cl<sub>2</sub>: abaxial leaf surfaces show broncening
2. O<sub>3</sub>, NO<sub>2</sub>: adaxial leaves appear silvery
3. HF and SO<sub>2</sub>: margins and tips of leaves appear necrotic.

These symptoms are rather general and are seldom visible as pure effects. Subthreshold, long-term exposures may lead to latent, chronic effects that are scarcely distinguishable from mineral deficiencies or pathological events brought about by infections (virus; fungi; see later discussion).

##### 2. Cellular and Subcellular Effects

Chloroplasts after different influences of various kinds of stresses show more or less characteristic effects in their thylakoid structures (see the contribution by Fink in Ref. 7). Typical of the influence of toxic air pollutants are the shrinking of the whole organelle and deformation of the thylakoids or swelling of grana structures. After exposure to subthreshold concentrations of air pollutants, the induction of ethene formation is mainly responsible for aging symptoms resembling natural senescence. Loss of the outer membrane, releasing lipid droplets (plastoglobuli) from the thylakoids, together with the appearance of rough endoplasmatic reticulum (an indication of increased metabolic activity such as the synthesis of lytic enzymes!) is another typical symptom. In conclusion one may say that the different trace gas emissions lead to relatively similar ultrastructural changes that may be reversible or irreversible, depending on the species and the circumstances of exposure described.

## B. Basic Chemical Reactions Underlying Toxic Activities

Basic chemical reactions of the most prominent potentially toxic trace gases are discussed in the following.

At the biological level it is almost impossible to differentiate between the individual influences under natural conditions since there is no single influence of one component of air pollutants on aerobic cells. Nevertheless, we discuss separately the influences of SO<sub>2</sub>, NO<sub>x</sub>, ozone, and peroxyacetyl nitrate (PAN) on important target molecules.

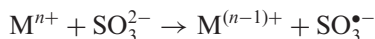
### 1. Dark Reactions: SO<sub>2</sub> and Derivatives

SO<sub>2</sub> is produced during combustion of organic materials, especially of coal. SO<sub>2</sub> and HSO<sub>3</sub><sup>-</sup> are involved in radical chain processes. HSO<sub>3</sub><sup>-</sup> is a reductant that, however, can accelerate oxidative processes by reducing peroxidic bonds, thus producing anions and radicals. Principally, SO<sub>2</sub> or HSO<sub>3</sub><sup>-</sup> preferentially reacts with the following types of molecules:

1. Aldehydes and ketones, in which hydroxysulfonates are formed, which in turn are inhibitors of several enzymes
2. Olefines, in which sulfonic acids are formed and the double bond is lost
3. Pyrimidines under formation of dihydrosulfonates, which may have mutagenic effects
4. Disulfides under formation of S-sulfonates, in which the S-S-bridge is split
5. Superoxide (O<sub>2</sub><sup>•-</sup>) under formation of the very reactive disulfite radical:

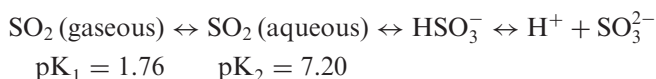


In this reaction, superoxide may be substituted by transition metal ions:



Most of the physiological effects can be induced by hydrogen abstraction as the primary reaction. None of the reactions mentioned, however, seems to be exclusively responsible for the observed toxic effects or symptoms. Of special importance is certainly the reaction with preformed hydroperoxides (see later discussion). SO<sub>2</sub> is a highly water-soluble gas that

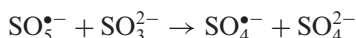
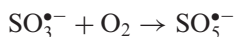
is rapidly hydrated, forming sulfite (16):



Sulfite modifies the cellular energy metabolism in mammalian tissues by decreasing the cellular adenosine triphosphate (ATP) pool; this may be due to changing the function of pyridine and flavin nucleotides (17).

If sulfite is not, as in animals, metabolized via sulfite oxidase, it may be oxidized via radical chain mechanisms. In the presence of oxygen, a series of  $\text{SO}_x^-$  and oxygen radicals are generated (see later discussion). These reactions induce lipid peroxidation and numerous connected reactions.

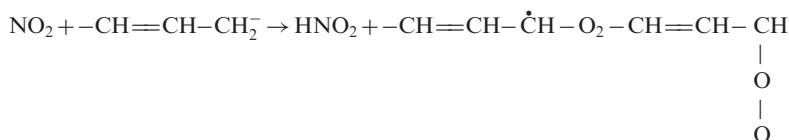
$\text{HSO}_3^-$  is a powerful initiator of several radical chain processes such as lipid peroxidation. Under aerobic conditions, the sulfate radical is generated from the sulfite radical:



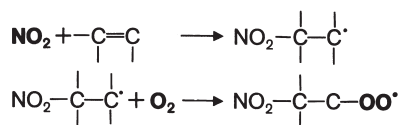
Both radical anions may be involved in destructive reactions initiated by  $\text{SO}_2$ . In clean air,  $\text{SO}_2$  is present in concentrations lower than  $20 \mu\text{g}/\text{m}^3$ . In heavily polluted areas close to coal power plants concentrations up to  $1200 \mu\text{g}/\text{m}^3$  may be observed. Conversion factors for  $\text{SO}_2$  are as follows:  $1 \text{ ppm} = 2670 \mu\text{g}/\text{m}^3$ ;  $\text{ppb} = 2.67 \mu\text{g}/\text{m}^3$ .

## 2. Dark Reaction: The Nitroxides, $\text{NO}_x$ : NO, $\text{NO}_2$

Both NO and  $\text{NO}_2$  are free radicals. Because of their unpaired electrons several one-electron reactions can be initiated in which the attack of olefinic structures (double bonds) is one characteristic. The first reaction is most probably the abstraction of a hydrogen atom from an allylic carbon atom forming a C-centered radical, which may add atmospheric oxygen according to



Addition reactions can also be observed, as shown in Scheme 3.

**Scheme 3** Reactivity of nitrogen dioxide.**Table 8** Bimolecular Reaction Constants for the Reaction of NO<sub>2</sub> with Carotenoids and Phenolics in Comparison to Other Radical–Radical Interactions

	Reaction	Type <sup>a</sup>	k [M <sup>-1</sup> S <sup>-1</sup> ]
1	2NO <sub>2</sub> <sup>•</sup> ⇌ N <sub>2</sub> O <sub>4</sub>	RR	4.5 × 10 <sup>8</sup>
2	N <sub>2</sub> O <sub>4</sub> (+H <sub>2</sub> O) → NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> + 2H <sup>+</sup>		1.0 × 10 <sup>3</sup>
3	NO <sub>2</sub> <sup>•</sup> + O <sub>2</sub> <sup>•-</sup> → O <sub>2</sub> NOO <sup>-</sup>	RR	4.5 × 10 <sup>9</sup>
4	NO <sub>2</sub> <sup>•</sup> + HO <sup>•-</sup> → NO <sub>3</sub> <sup>-</sup> + H <sup>+</sup>	RR	4.5 × 10 <sup>9</sup>
5	NO <sub>2</sub> <sup>•</sup> + HO <sup>•-</sup> → ONOOH	RR	4.5 × 10 <sup>9</sup>
6	NO <sub>2</sub> <sup>•</sup> + ascorbate → 2NO <sub>2</sub> <sup>-</sup> + ascorbate radical anion	ET	3.5 × 10 <sup>7</sup>
7	NO <sub>2</sub> <sup>•</sup> + caffeic acid → NO <sub>2</sub> <sup>-</sup> + [caffeic acid] <sup>•+</sup>	ET	8.6 × 10 <sup>8</sup>
8	NO <sub>2</sub> <sup>•</sup> + ferulic acid → NO <sub>2</sub> <sup>-</sup> + [ferulic acid] <sup>•+</sup>	ET	7.4 × 10 <sup>8</sup>
9	NO <sub>2</sub> <sup>•</sup> + sinapic acid → NO <sub>2</sub> <sup>-</sup> + [sinapic acid] <sup>•+</sup>	ET	7.2 × 10 <sup>8</sup>
10	NO <sub>2</sub> <sup>•</sup> + kaempferol → NO <sub>2</sub> <sup>-</sup> + kaempferol <sup>•</sup> + H <sup>+</sup>	ET	3.4 × 10 <sup>8</sup>
11	NO <sub>2</sub> <sup>•</sup> + arachidonate ion → products	?	1.0 × 10 <sup>6</sup>
12	NO <sub>2</sub> <sup>•</sup> + β-carotene → NO <sub>2</sub> <sup>-</sup> + β-carotene radical	ET	1.1 × 10 <sup>8</sup>
13	NO <sub>2</sub> <sup>•</sup> + canthaxanthin → NO <sub>2</sub> <sup>-</sup> + [canthaxanthin] <sup>•+</sup>	ET	1.2 × 10 <sup>7</sup>
14	NO <sub>2</sub> <sup>•</sup> + astaxanthin → NO <sub>2</sub> <sup>-</sup> + [astaxanthin] <sup>•+</sup>	ET	1.3 × 10 <sup>7</sup>
15	NO <sub>2</sub> <sup>•</sup> + lutein → NO <sub>2</sub> <sup>-</sup> + [lutein] <sup>•+</sup>	ET	1.6 × 10 <sup>7</sup>
16	NO <sub>2</sub> <sup>•</sup> + zeaxanthin → NO <sub>2</sub> <sup>-</sup> + [zeaxanthin] <sup>•+</sup>	ET	2.1 × 10 <sup>7</sup>
17	NO <sub>2</sub> <sup>•</sup> + lycopene → NO <sub>2</sub> <sup>-</sup> + [lycopene] <sup>•+</sup>	ET	1.9 × 10 <sup>7</sup>

<sup>a</sup>Reaction types: RR, radical–radical interaction; ET, electron transfer.

Source: Ref. 18.

Several secondary plant constituents acting as antioxidants react rapidly with NO<sub>2</sub> and are thus involved in its detoxification, yielding different products with different velocities.

Table 8 lists some reaction constants. The primary product of combustion is NO. NO and NO<sub>2</sub> are tightly connected with each other in atmospheric chemical reactions via a very complicated chain of reactions involving ozone and a vast amount of volatile organic molecules (VOCs). Therefore calculating activities or concentrations of only NO or NO<sub>2</sub> is not very meaningful in vivo.

**Table 9** Dose–Response Data for NO<sub>2</sub> for Plants, Humans and Animals<sup>a</sup>

Effects on humans and animals	No effects in healthy persons in concentrations up to 1900 µg/m <sup>3</sup> ; asthmatics, especially under strain, may recognize respiratory restrictions at below 950 µg/m <sup>3</sup> ; increase of respiratory infections at concentrations above 5000 µg/m <sup>3</sup> were reported for animals; no indication for lung cancer after chronic exposition
Effects on plants	No negative effects on plants at below 4000 µg/m <sup>3</sup> ; cooperation with NH <sub>4</sub> <sup>+</sup> as fertilizer; under acid conditions csoil losses of Mg <sup>2+</sup> and Ca <sup>2+</sup> ions; changes in ecological equilibria due to altered competitive behavior, especially in N-intolerant ecotypes such as boglands

<sup>a</sup>Concentrations up to 850 µg/m<sup>3</sup> (1/2 h means) may be measured in urban areas.

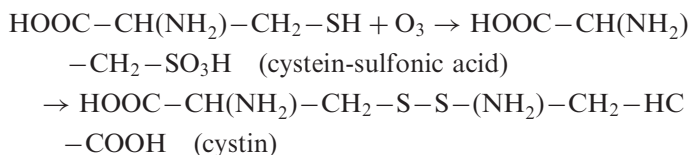
In Table 9 general dose–response effects of NO<sub>2</sub> on plants and animals are compared. The conversion factors for NO<sub>2</sub> are as follows:

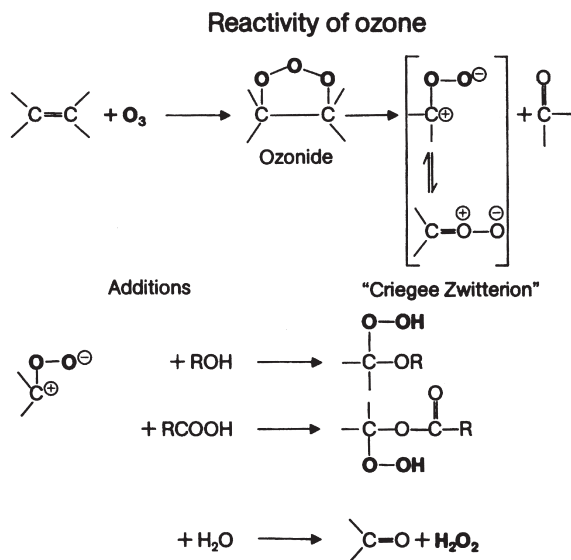
$$1 \text{ ppm} = 1910 \text{ µg/m}^3; \quad 1 \text{ ppb} = 1.91 \text{ µg/m}^3$$

### 3. Dark Reactions: Ozone and Peroxides *Peroxyacetyl Nitrate*

*Ozone.* Ozone is formed from oxygen and NO<sub>x</sub> catalyzed by VOC in a chain of atmospheric events (see previous discussion; also see Ref. 15). Ozone is a zwitterion (dipolar ion) [(-)O–O(+)=O] and thus a very reactive molecule as a result of its ionic resonance structure. Having a standard redox potential of about 2 volts, it is a strong oxidant. Characteristic chemical reactions with biochemical relevance are the oxidative splitting of olefinic bonds and the reactions with thiols. Ozonization of olefines yields intermediary ozonides, which in turn decompose into ketones and other products probably via the so-called Criegee zwitterions. The resonance structures allow additions of carboxylic acids, alcohols, and water, finally yielding different peroxidic products (Scheme 4).

Thiol oxidation leads to sulfonic acids or disulfides such as cystin:





**Scheme 4** Reactions of ozone with double bonds and additions to the zwitterion.

Similarly, glutathione and proteinic SH groups are oxidized, thus drastically changing the antioxidative power and cellular redox balances and impairing enzymic functions: the oxidation of SH groups is responsible for inhibition of the lipid synthesis in mitochondria and microsomes: acyl-coenzyme A-thioesterases (acyl-CoA-thioesterases), acyl-CoA-thiokinases, and acyl-transferases are blocked, detectable as inhibition of the glycerol-3-phosphate acylation (19). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis, is also inactivated, whereas the oxidation of SH groups in the active center is responsible for the rapid loss of function. In addition, peripheric SH groups, tryptophan, histidine, and methionine groups are oxidatively damaged (20), thus provoking proteolytic degradation. Ozone inactivates  $\alpha$ 1-proteinase inhibitor via methionine oxidation (21), and lysozyme via the conversion of tryptophan into *N*-formyl kynurenine (22). Because of its ionic resonance structure ozone is more water-soluble than oxygen. Therefore hydrophilic compartments may be the main site of its reaction. Under acidic conditions ozone is quite stable, under basic conditions it decomposes into molecular oxygen. At 22°C 100 ppm ozone on a water surface yields micromolar solutions. It can be calculated that up to 10 moles of ozone is dissolved in 55 moles H<sub>2</sub>O. In the presence of aromatic compounds O<sub>3</sub> is decomposed into OH<sup>•</sup> radical-type oxidants. In the presence of several cellular compounds such as cysteine,

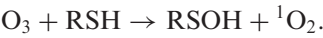


**Table 10** Relative Sensitivities to Ozone: Dose–Response Effects for Plants, Humans, and Animals<sup>a</sup>

Effects on humans and animals	Sensitive persons (not identical to asthmatics) may undergo reversible respiratory restrictions (ca. 4%–8% reduction at 200–400 µg/m <sup>3</sup> ); during strain respiratory functions change concentrations above 160 µg/m <sup>3</sup> ; adaptations after repeated exposures have been observed; headache, coughing, and throat irritations were individually reported at concentrations above 200 µg/m <sup>3</sup> ; in mice, increased susceptibilities to viral infections (>160 µg/m <sup>3</sup> ) were observed; no indication for carcinogenic potentials
Effects on plants	Losses in crop yields and growth reductions up to 10% in sensitive plants (7 h up to 70 µg/m <sup>3</sup> ) were reported

<sup>a</sup>Concentrations up to 450 µg/m<sup>3</sup> (1/2 h means) may be measured close to but outside urban or industrial centers.

methionine, reduced glutathione (GSH), ascorbate, and NAD(P)H, ozonization also yields singlet oxygen:



Dependent on the concentrations of NO, aromatic compounds, and high light intensities, O<sub>3</sub> can increase strongly: O<sub>3</sub> level up to 400–500 µg/m<sup>3</sup> can be reached on hot and sunny days. Average concentrations in rural areas during summer days may be 20–80 µg/m<sup>3</sup>, depending on time of day and altitude. In summer 1994 episodes with 150–250 µg/m<sup>3</sup> O<sub>3</sub> were frequently observed in Germany. Conversion factors for O<sub>3</sub> are as follows:

$$1 \text{ ppm} = 2000 \text{ µg/m}^3 \quad 1 \text{ ppb} = 2 \text{ µg/m}^3$$

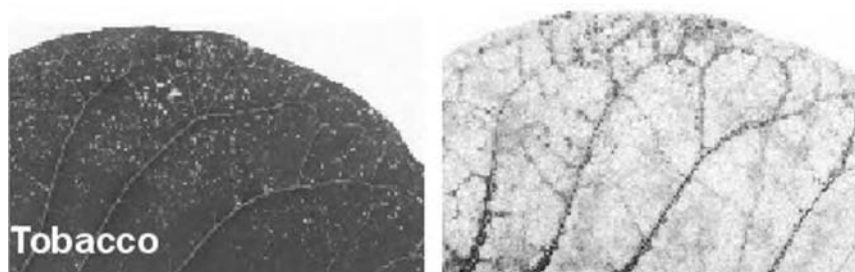
Relative sensitivities of plants, humans and animals towards ozone are given in Table 10.

*Critical Levels for Ozone.* Adverse effects of elevated ozone levels on plant productivity and competitiveness have been described, and herbaceous plants have been defined as considerably more sensitive than tree species (23,24). Currently, critical levels for ozone in the United States are all episodes that exceed 60 nl/l (SUM06). In Europe, critical levels for ozone injury in plants have been established by using accumulated hourly doses over a threshold of 40 nl/l (AOT40) as a basis (24). This approach neglects ozone levels below 40 nl/l, which do not cause visible injury or growth reduction in the most sensitive plants, e.g., the tobacco cultivar Bel W3. The

proposed AOT40 values for crops ( $3,000 \text{ nl l}^{-1} \text{ h}^{-1}$ ) and trees ( $10,000 \text{ nl l}^{-1} \text{ h}^{-1}$ ) are intended to indicate ozone risks on plants rather than quantifying ozone impacts. Both values are often exceeded in Central Europe and North America, suggesting that ozone is a potential threat for plants. However, the correlation of SUMO6 and AOT40 to the degree of visible symptoms and to growth reduction is variable. Therefore, recent approaches for critical ozone levels are based on the ozone flux into the leaves. As the stomatal pathway is the predominant pathway (>99%) of ozone uptake, all factors, such as humidity and temperature, that modify the stomatal aperture affect ozone influx, resulting in a nonproportional relation between external and internal ozone doses (24).

Once taken up through stomata, ozone is rapidly decomposed to secondary reactive oxygen species (ROS), such as superoxide anion radicals ( $\text{O}_2^{\bullet-}$ ),  $\text{H}_2\text{O}_2$ , hydroxyl radicals ( $\text{OH}^{\bullet}$ ), and other species in the leaf apoplast (25). These ROS can further react with components of the cell wall and the plasma membrane (26). It is therefore assumed that the internal ozone dose in the gas space of leaves is close to zero (27). Evidence for ozone-derived ROS was obtained in an electron paramagnetic resonance spectrometry study (28) in which a signal resembling that of  $\text{O}_2^{\bullet-}$  and with putative chloroplastic localization occurred during and up to 15 min after the end of ozone exposure.

*Sensitivity of Herbaceous and Forest Plants.* Average summer ozone concentrations are sufficient to provoke cell death and/or chlorotic symptoms on leaves of distinct sensitive species and cultivars, although the majority of plants do not exhibit visible leaf injury (29). The “classical” ozone symptoms were first described as “weather flecks” of tobacco in the 1920s. They occurred as sharply defined lesions on the adaxial side of the leaf resulting from the death of groups of palisade cells (see Fig. 1; also see

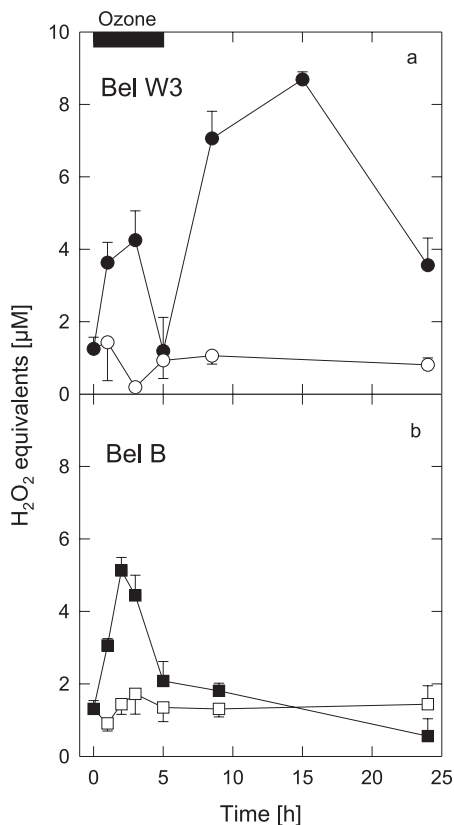


**Figure 1** Typical ozone symptoms on the ozone biomonitor plant, tobacco Bel W3, after 24 h: (left) sites of  $\text{H}_2\text{O}_2$  accumulation 8 h after the onset of a 5-h exposure period to  $150 \text{ nl/l}$  ozone (right).

Ref. 29). Weather flecks occurred under distinct weather conditions, i.e., during sunny days followed by rain periods. Breeding experiments revealed a genetic background of sensitivity for weather fleck symptoms. The most important biomonitor plants, the “hypersensitive” tobacco cultivar Bel W3 and its tolerant counterpart Bel B, stem from breeding programs in the 1950s. Both cultivars have been used in biomonitor programs worldwide for more than 40 years. In addition to other crop species and cultivars, a survey of native European plants revealed that several dicot species, including *Malva sylvestris* L., *Rumex crispus* L., and *Rumex obtusifolius* L., also respond to elevated ozone levels with cell death of leaf mesophyll cells. Thus, sensitivity to ozone on one hand and resistance to nonpathogens and avirulent pathogens on the other are both characterized by cell death in (middle-aged) leaves, suggesting that similar processes underlie these abiotic and biotic stress responses.

*Amplification Reactions for Ozone-Induced Cell Death: The Oxidative Burst.* Work in recent years has indicated that, in addition to direct ROS formation from ozone, this air pollutant does induce an oxidative burst in model plants, resulting in the accumulation of ROS and activation of signaling pathways that overlap with the hypersensitive response (HR) against pathogens (26,30,31). The oxidative burst is a massive, rapid, and transient activation of oxidative metabolism immediately after exposure to certain abiotic and biotic stress factors. By analogy with the respiratory burst, a primary response of mammalian macrophages and neutrophils to invading pathogens can be seen (see Chapter 10). The oxidative burst is found as one of the earliest events in the HR to pathogens, a suicide program around the invasion site, involving localized host cell death and transcriptional activation of defense genes in both the challenged and surrounding cells.

The ozone bioindicator tobacco Bel W3 responded with biphasic  $\text{H}_2\text{O}_2$  accumulation during phase I and after the exposure period (phase II), during postcultivation in pollutant-free air (Fig. 2). The ozone-tolerant counterpart Bel B exhibited a phase I peak only during the exposure period. It was concluded that ozone effects are amplified by cellular production of ROS only in the sensitive cultivar (reviewed in Ref. 31). Similarly, ultrastructural analysis of birch leaf cells revealed that  $\text{H}_2\text{O}_2$  accumulation starts in the cell wall and at the plasma membrane and proceeds into the postcultivation period. Further studies showed (31) that an ozone-triggered oxidative burst of plant origin is a common feature of ozone-sensitive tomato cultivars, *Arabidopsis* accessions, and wild plant species. The predominant type of ROS (superoxide or  $\text{H}_2\text{O}_2$ ) that accumulated differed among species, cultivars, and accessions but coincided with cell death that



**Figure 2** Oxidative burst in ozone-exposed tobacco Bel W3 (ozone-sensitive) and Bel B (tolerant) during (phase I) and after (phase II) ozone exposure during postcultivation in pollutant-free air.

occurred considerably later (Fig. 1b). Finally, ROS accumulation as well as lesion development were substantially reduced by inhibitors of flavin-containing oxidases, peroxidases (PODs), and protein kinases. In summary, these results provided evidence for ozone as a trigger of ROS-producing activities but did not allow assignment of (a) specific enzyme(s) for ROS production. Given the diverse responses of the species and cultivars analyzed so far, it seems probable that more than one enzymatic source of ROS formation is operating in different species.

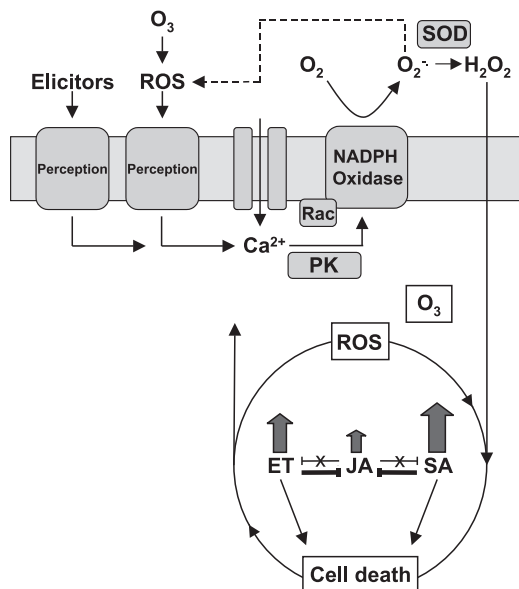
Four main enzymatic processes may be involved in apoplastic ROS production in monocot and dicot species: plasma membrane NAD(P)H oxidase, extracellular pH-dependent POD, oxalate oxidase, as well as

diamine and polyamine oxidases (32,33). In classical studies, the oxidative burst in potato was characterized as NADPH-dependent  $O_2^{\bullet-}$  production (reviewed in Ref. 34). It was postulated that a novel superoxide producing NAD(P)H oxidase in the membranes of potato and tobacco tissues is activated during infection, resulting in cell death and defense response activation. Plant homologs of the catalytic subunit of neutrophil NADPH oxidase, *gp91<sup>phox</sup>*, have been isolated in the meantime from various plants, including rice, *Arabidopsis* spp., tobacco, tomato, potato, and parsley. The homology at the deduced amino acid level is rather low (between 25% and 35%), but the important sequence features, e.g., flavin adenine dinucleotide (FAD) as well as NADPH binding sites, conserved histidine residues for heme binding as well as six putative transmembrane-spanning domains. In contrast to the mammalian NADPH oxidase in neutrophils, all plant homologs known so far show an N-terminal extension with one or two putative  $Ca^{2+}$  binding motifs, suggesting that direct regulation of the enzyme by  $Ca^{2+}$  ions is possible in plants (33).

Reduced nicotinamide adenine dinucleotide phosphate oxidase was postulated to be involved in the generation of the ozone-triggered oxidative burst in tobacco Bel W3. Two NADPH oxidase genes, *NtrbohD* and *NtrbohF*, were isolated from the ozone-sensitive tobacco Bel W3. Whereas isoform F was constitutively present at low levels in all tissues, *NtrbohD* transcripts as well as NADPH oxidase activity were induced by ozone when plants were exposed in climate chambers and in the field. The induction kinetics showed biphasic behavior with maxima after 2 h (phase I) and 6 h (phase II); hence, NADPH oxidase activity could be responsible, at least in part, for the biphasic ozone-induced oxidative burst in tobacco (Fig. 3).

The family of peroxidase (POD) proteins (donor,  $H_2O_2$  oxidoreductases; EC 1.11.1.7) has diverse functions in lignification, wound responses, pathogen attack, and growth regulator action. Whereas the majority of proteins function in the removal of  $H_2O_2$ , a subfamily of secretory POD is able to produce  $H_2O_2$  through compound III, a superoxide binding intermediate, requiring a suitable reductant as well as alkaline pH (33). Several PODs are localized to the vacuolar and apoplastic compartments; POD is the major enzymatic source of  $H_2O_2$  accumulating in *Phaseolus vulgaris* L. (French bean), and a cell-wall-located POD colocalized to  $H_2O_2$  accumulation at bacterial infection sites. It is therefore possible that cell wall PODs are also involved in the generation of the oxidative burst under ozone and pathogen stress.

*Amplification Reactions for Ozone-Induced Cell Death: Ethylene and Salicylic Acid.* As ROS are necessary but not sufficient to trigger cell



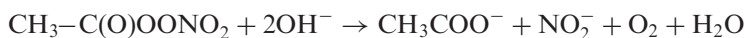
**Figure 3** Model of the oxidative cell death cycle in ozone-exposed plants. This cycle includes amplification of ROS accumulation by NADPH oxidase or other ROS-producing enzymes, salicylic acid (SA), and ethylene (ET) whereas jasmonate (JA) leads to lesion containment. CDPK, calium-dependent protein kinase; SOD, superoxide dismutase; ROS, reactive oxygen species; NADPH, RAC (low molecular weight GTPase), PK, protein kinase.

death, unraveling the identity of other players in the initiation and propagation of ozone-induced cell death is of utmost importance. Reactive oxygen species, salicylic acid, ethylene, and NO were postulated to cooperate in the so-called oxidative cell death cycle in pathogen-infected and ozone-treated plants (31,35). Ethylene is the earliest biochemical response to ozone in all species investigated so far and is closely linked to ozone sensitivity. Salicylic acid is a major phenolic compound involved in defense gene activation and initiation of cell death during oxidative stress. Levels of free salicylic acid were induced before ROS accumulation and cell death took place in ozone-sensitive *Arabidopsis* spp. and tobacco. On the other hand, salicylic acid levels were only weakly induced in ozone-tolerant tobacco.

It was postulated in 2003 that ozone itself or ozone-derived ROS activate an amplification loop of cellular ROS production in sensitive plants (31; Fig. 3). In addition to other enzymatic sources, NADPH oxidase may be responsible for extracellular ROS generation in the ozone-triggered

oxidative burst. Other key players in the initiation and propagation of cell death are NO, lipid-based molecules (LOOH), salicylic acid, and ethylene, which operate in a self-amplifying cycle, whereas jasmonate may lead to lesion containment. Accordingly, ozone seems to trigger the pathogen-defense pathway erroneously, leading to HR-type cell death in crop and native plant species. The model in Fig. 3 predicts that plants can resist oxidative stress as long as the ethylene and salicylic acid pathways are not activated in excess or are inhibited by jasmonate. In addition, both ethylene and salicylate pathways have to be active to propagate ozone-induced cell death. In the absence of the oxidative stress, the balance between the signaling pathways inhibits the activation of the cell death-promoting effect of ethylene and salicylate. During oxidative stress produced by ozone exposure, but also by other abiotic and biotic factors, the balance between the signaling pathways is altered toward ethylene and salicylate induction to allow cell death to occur. It seems that the biosynthetic kinetics of ethylene, salicylate, and jasmonate is critical for the regulation of the signaling balance and the concomitant cell death in ozone-exposed plants.

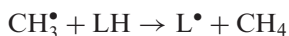
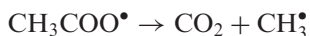
*Peroxides (Peroxyacetyl Nitrate).* Different peroxides are present in polluted air; in this respect peroxyacetyl nitrate (PAN) and  $\text{H}_2\text{O}_2$  are of major importance. In centers of “photosmog” such as the Los Angeles region PAN may yield up to 5%–20% of the corresponding ozone concentrations. Besides PAN, other peroxides such as peroxypropionyl, peroxybutyryl, and peroxybenzyl nitrate have to be considered as plant-toxic species. At physiological pH (7.2) PAN has a half-life of 4.4 min. Its decay is base-catalyzed, mainly yielding acetate and nitrite:



Peroxyacetyl nitrate may react with the following chemicals and biochemicals: Thiol groups, especially in enzymes, such as the SH groups of glucose-6-phosphate (Glu-6-P) dehydrogenase; the substrates, Glu-6-P and nicotinamide adenine dinucleotide (NAD), partially prevent SH oxidation. Smaller S-containing molecules are also oxidized: methionine is converted into methionene sulfoxide and lipoic (thioctic-) acid, and coenzyme A are converted into the corresponding disulfides. In the case of glutathione 1 mole PAN converts 3 moles of SH groups.

1. Peroxyacetyl nitrate reacts with NADPH; the oxidized compounds (NAD, nicotinamide adenine dinucleotide phosphate [NADP]) do not seem to react further with PAN.

2. Peroxyacetyl nitrate reacts with olefins, forming epoxides and further products of lipid peroxidation:



3. Reactions with primary amines result in their acetylation:



Conversion factors for PAN are the following: 1 ppm PAN = 4370  $\mu\text{g}/\text{m}^3$ ; 1 ppb PAN = 4.37  $\mu\text{g}/\text{m}^3$ .

4. Trace Gases not Directly Causing Damage via Free Radical Mechanisms: Ethene and Hydrogen Fluoride

*Ethene.* Ethene (formerly ethylene) as a retarding phytohormone is, as abscisic acid is, an antagonist to the growth stimulators auxins and gibberellins. It induces fruit ripening, stem thickening, senescence, and increased resistance, and general responses to all kinds of stress and wounding. Under natural conditions (fruit ripening, mild stress) ethene is formed from methionine via S-adenosyl methionine (SAM) and 1-amino-cyclopropane carboxylic acid (ACC), from which it is released oxidatively (Fig. 4).

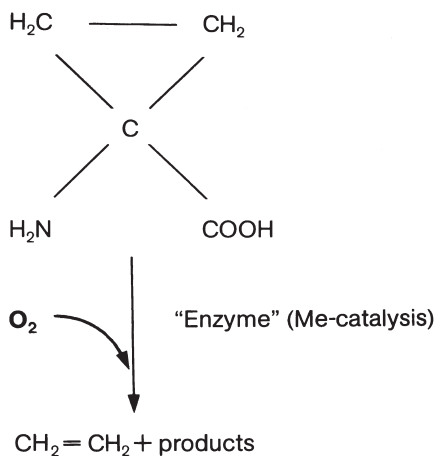
Under acute stress situations such as freezing and mechanical wounding small amounts of ethene may originate from the oxidative breakdown of omega-3 polyunsaturated fatty acids such as linolenic acid. Together with ethane, ethene is derived from the omega-1 and -2 fragment of the fatty acid under the catalysis of copper ions (36).

In the absence of copper ions ethane is the normal characteristic product of chaotic lipid peroxidation, which generally is determined as malondialdehyde by means of the thiobarbituric acid (TBA) reaction. Such reactions occur in the photosystems after limitation of electron transport (ET) as outlined in Chapter 2.

1. Blocking the electron transport, for example by the ET inhibitor dichlorophenyl dimethyl urea [DCMU] (an experimental herbicide) yields singlet oxygen ( $^1\text{O}_2$ ) via triplet chlorophyll ( $^3\text{Chl}$ ) and a photodynamic reaction of type II 1-amino-cyclopropane-1-carboxylic acid (ACC). Photodynamic reactions of type I, on the other hand, may produce free radicals such as  $^\bullet\text{OH}$  or C-centered.



## ACC oxidation

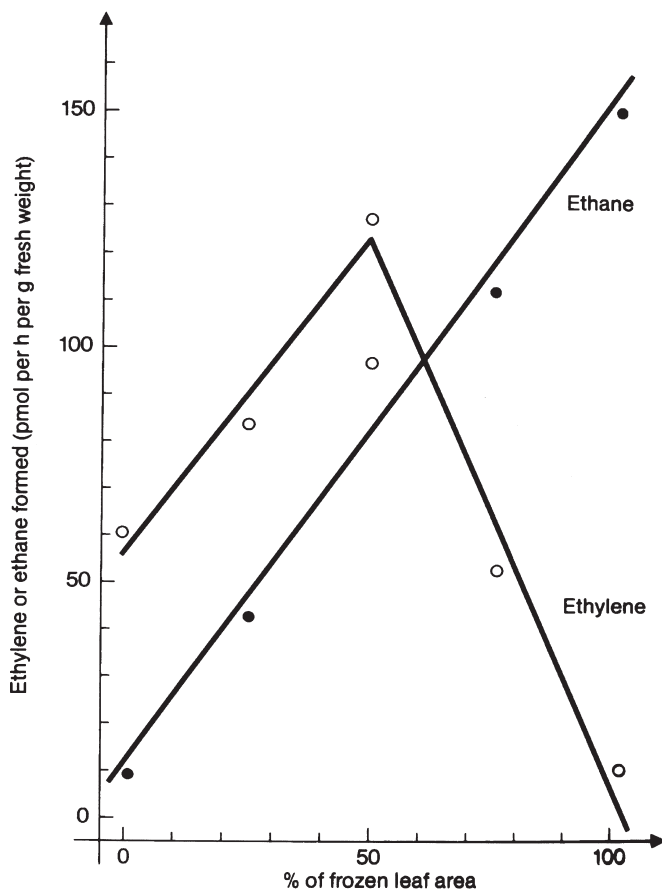


**Figure 4** Ethene formation from 1-aminocyclopropane-1-carboxylic acid (ACC).

2. Singlet oxygen or free radicals derived from oxidized unsaturated fatty acids (e.g.,  $\alpha$ -linolenic acid), yielding ethane. Wounding (point freezing) induces lipid peroxidation in the dark (enzymic via lipoxygenases [LOXs]), producing ethane with increasing wound area, indicating that this reaction is chaotic and takes place in the decompartmentalized tissue. Ethene formation, in contrast, in the same tissue, also depends on wounding but occurs in substantial amounts only in intact tissues adjacent to the wound (optimally at 50% wounding) and is stopped as soon 100% of the tissue is decompartmentalized (Fig. 5).

In these more or less uncatalyzed wound reaction pathways, which also release the so-called green odor (freshly cut grass!), products such as *cis*- and *trans*-hexenal and hexenal, lipid peroxidations, phenol oxidations, and pigment co-oxidations, occur simultaneously, leading to more or less toxic substances. Thus, a plethora of free radicals, aldehydes, and *o*-quinones may serve as pathogen defense. In addition polymerization and condensation products of phenols and lipids such as the melanins and lipofuscins are involved in wound cleaning and closure (see also Chapter 2).

Ethene is released in traces by all burning processes and is thus increased in industrial areas with heavy traffic. In the United States annually



**Figure 5** Ethane and ethene formation in wounded leaf tissue. (From Ref. 37.)

combustion gases  $10\text{--}30 \times 10^9$  kg are released by industry and traffic and combustion gases  $2 \times 10^7$  kg by plants themselves. Ethene (mainly together with ethane!) is released by plants under stress (wounding,  $\text{SO}_2$ ,  $\text{O}_3$ ) or partial freezing (37) and may interact with oxidants such as ozone, forming reactive formaldehyde; with  $\text{NO}_x$  it may contribute to the formation of ozone. As a general rule in estimating ethene as a plant effector one may use the following ethene concentrations in the air:

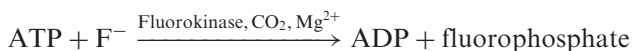
10 ppb	( $11.6 \mu\text{g}/\text{m}^3$ )	no effect
100 ppb	( $116 \mu\text{g}/\text{m}^3$ )	half maximal effects
1–10 ppm	( $1160\text{--}11600 \mu\text{g}/\text{m}^3$ )	saturation
	$= 1.16\text{--}11.6 \text{ m}^3$	

In rural areas one has to envisage concentrations of  $3.5\text{--}12\text{ }\mu\text{g}/\text{m}^3$ , whereas in cities values of  $120\text{ }\mu\text{g}/\text{m}^3$  may be reached, thus allowing the induction of processes of senescence and acceleration of ripening. On the other hand increased resistance to fungal diseases in these areas may be explained by the induction of defense processes such as the shikimate pathway via induction of key enzymes, Phenylalanine ammonia lyase (PAL) and cholinesterase (CHS), to name just two. This phenomenon, namely, the induction of defense reactions against other stress factors by subthreshold stressors such as ozone, is part of an integrated system, called *systemic acquired resistance* (SAR), and may operate via the hormone ethene (see previous discussion). Conversion factors for  $\text{C}_2\text{H}_4$  are as follows:  $1\text{ ppm} = 1160\text{ }\mu\text{g}/\text{m}^3$ ;  $1\text{ ppb} = 1.16\text{ }\mu\text{g}/\text{m}^3$ .

*Hydrogen Fluoride.* Fluoride causes necroses on the tips and edges of leaves (tip burn), where the symptoms underlie a typical threshold phenomenon. Uptake of hydrogen fluoride (HF) is passive, and the transport follows the transpiration stream. In oats it accumulates in the following organs in decreasing order:

Cell wall > chloroplasts > soluble protein > mitochondria  
> microsomal fraction

Hydrogen fluoride is no longer a major threat to plants, as it was in former times in proximity to glass factories that released HF in substantial amounts from their smelters. In the series of important gaseous plant poisons such as PAN and other peroxides,  $\text{SO}_2$ , ozone, and  $\text{NO}_x$ , HF is not reactive via free radical mechanisms, but acts rather as an inhibitor of enzymes such as enolase, succinate dehydrogenase, phosphoglucomutase, and generally phosphate- and ATP-converting enzymes. The inhibition may, at least in part, be due to the formation of fluorophosphate according to

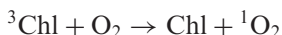
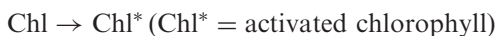


which is the actual inhibitor in some cases. One major pathway of inhibition is the poisoning by fluorocitrate of the aconitase activity in the tricarboxylic acid cycle via incorporation into fluoroacetate and thus fluorocitrate. Citrate accumulates as a consequence and in turn has a feedback effect (Pasteur effect) on pivotal elements of intermediary metabolism and thus on the distribution of carbon within the main catabolic pathways, namely, pentose phosphate pathway and glycolysis. Conversion factors for HF are the following:  $1\text{ ppm} = 830\text{ }\mu\text{g}/\text{m}^3$ ;  $1\text{ ppb} = 0.83\text{ }\mu\text{g}/\text{m}^3$ .

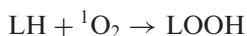
### C. Light-Driven Reactions and Formation of Reactive Oxygen Species

As humans and animals may, plants under several conditions may suffer from stress, although the conditions by definition may be completely different from those in animal systems (38). Besides drought, flooding, frost, heat, soil chemicals, and many others, air pollution represents one major stressor. As outlined in more detail in Chapter 2, under continuous light most of the conditions mentioned yield an overreduction of the photosynthetic electron transport chain due to limited NADPH reoxidation. Thus, under high-light intensities air pollutants have much stronger effects as compared to those under low light conditions.

Under the influence of air pollutants and conditions with limited oxidized nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) in the stroma of the chloroplasts (i.e., strong light, transport limitations, ATP uncoupling, inhibition of Calvin cycle activities, and others) reductive and photodynamic oxygen activations are measurable. Inhibited CO<sub>2</sub> fixation due to stomatal closure or inhibition of the Calvin cycle due to blocked translocation of photosynthetic product is especially likely to lead to this limited reoxidation of NADPH + H<sup>+</sup>. These conditions result in the overreduction of the electron transport chain mentioned and a lack of the potential of charge separation after chlorophyll activation in the light. Thus, inhibited energy transfer supports photodynamic reactions of type II, generating singlet oxygen (39):



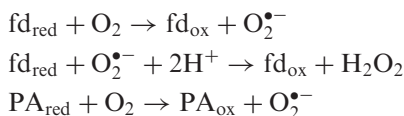
The electrophilic nature of singlet oxygen results in chemical reactions with double bonds or other electron rich regions of molecules. Singlet oxygen may eventually be quenched by carotenoides or  $\alpha$ -tocopherol in the thylakoid membranes or directly react with unsaturated fatty acids (LH), forming hydroperoxides (LOOH) (40):



Membrane disruption due to lipid peroxidation is a common feature of photodynamic actions. Valenzo (41) reviewed different photomodifications of biological membranes with emphasis on singlet oxygen mechanisms. He concluded that effective membrane sensitization usually involves an

association of the sensitizer with the membrane. This happens especially in thylakoid membranes of the chloroplasts.

Limited NADP<sup>+</sup> availability also results in the formation of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> via overreduction of the electron transport system and subsequent electron channeling to oxygen. Oxygen may be reduced either by the primary acceptor (PA) of photosystem I, by reduced ferredoxin (fd<sub>red</sub>), or by both (42):

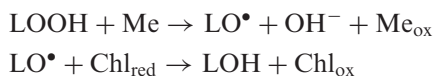


H<sub>2</sub>O<sub>2</sub> may be further reduced by reduced ferredoxin, forming ROS with properties similar to those of the hydroxyl radical (OH<sup>•</sup>):

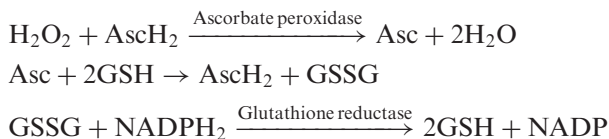


More details on these photosynthetic reactions are presented in Chapter 2.

Reductively and photodynamically generated ROS oxidatively degrade fatty acids in the membranes. The hydroperoxides resulting from fatty acid peroxidation decay via transition-metal-catalyzed reduction, forming alkoxyl radicals (LO<sup>•</sup>), which in turn co-oxidize chlorophyll and other pigments, initiating their bleaching:



Reactive oxygen species can be detoxified in the chloroplasts. Superoxide dismutation is catalyzed by copper zinc SOD, which is found in the stroma as well as bound to the thylakoids. Since chloroplasts are devoid of catalase, H<sub>2</sub>O<sub>2</sub> detoxification is brought about by the ascorbate (AscH<sub>2</sub>/Asc) and glutathione (2GSH/GSSG) redox cycle at the expense of NADPH<sub>2</sub> (Beck–Halliwell–Asada cycle, or water–water cycle, cf. Chapter 2):



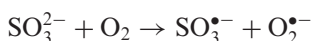
The whole process not only detoxifies H<sub>2</sub>O<sub>2</sub> but also decreases the NADPH<sub>2</sub>/ATP ratio.

Thus, lipid peroxidation and pigment-co-oxidation due to the generation of ROS take place only when the defense system of the chloroplast is overcharged and avoidance reactions are out of balance (see Chapter 2).

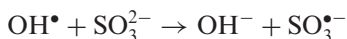
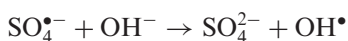
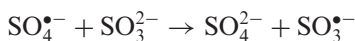
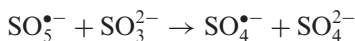
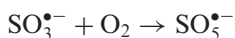
1. SO<sub>2</sub> in the Light

Plant damage caused by SO<sub>2</sub> is oxygen- and light-stimulated. Typical SO<sub>2</sub> effects are the rapid inhibition of photosynthesis before direct symptoms such as bleaching of chlorophyll can be observed. SO<sub>2</sub> or, in aqueous solution, sulfite (HSO<sub>3</sub><sup>-</sup>) can be oxidized in the presence of oxygen via a radical chain mechanism (Scheme 5).

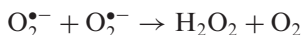
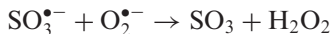
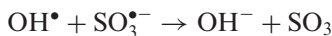
Initiation:



Propagation:



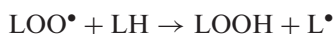
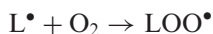
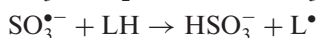
Termination:



**Scheme 5** Radical–radical interactions between sulfur oxides and oxygen.

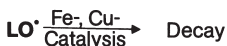
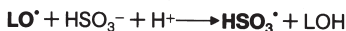
The initiation can be started by metal ions, UV irradiation, or enzymatic reactions. Free radicals generated during sulfite auto-oxidation attack cellular membranes via lipid peroxidation and subsequent chlorophyll bleaching. In addition lipid peroxidation is initiated by sulfite radicals generated during the reaction between sulfite and superoxide (16) (Scheme 6).

Initiation



**Scheme 6** Initiation and propagation of lipid (LH) peroxidation (forming LOOH) and pigment co-oxidation; LH, unsaturated fatty acid in lipid.

### $\text{HSO}_3^-$ as radical-propagating agent



**Figure 6** Chlorophyll bleaching by cooperation of fatty acid peroxides and bisulfite. (Adapted from Ref. 16.)

The bleaching of chlorophyll by lipid peroxidation is only observed if  $\text{HSO}_3^-$  is simultaneously present. Neither LOOH nor  $\text{HSO}_3^-$  alone can destroy the green color of chlorophyll. The interaction of bisulfite and LOOH in chlorophyll bleaching is shown in Fig. 6.

The  $\text{H}_2\text{O}_2$  generated is thought to be responsible for the inactivation of ascorbate peroxidase, glutathione reductase, and enzymes of the Calvin cycle, such as phosphatases.  $\text{SO}_2$  directly inhibits the translocation of photosynthetic products via disturbed phloem loading.

## 2. Ozone in the Light

Disturbed phloem loading is also observed after ozone exposure of higher plants and associated with an inactivation of the plasmalemma-bound adenosine triphosphatase (ATPase) (43). Secondary events lead to increased starch accumulation and finally bleaching of the photosynthetic pigments, evoking symptoms, which sometimes can be scarcely distinguished from virus infection (see Chapter 10).

Ozone further acts as an effective uncoupler of photophosphorylation (44). Studies with isolated chloroplasts showed that ozone blocks the energy transfer from the primary light acceptors to the reaction centers, initiating photodynamic reactions of type II, as mentioned. The significance of this observation remains to be established in vivo, since ozone probably cannot penetrate the outer chloroplast membrane. Aben and coworkers (45) showed that low-level ozone ( $120 \mu\text{g}/\text{m}^3$  8 h/day over 2 wk) can have a direct effect on the stomata and the photosynthetic system, causing decreased photosynthesis and stomatal conductance.

## D. Cooperative Effects of Gases

In our atmosphere single toxic trace gases rarely occur; in most cases complex mixtures (e.g., during photochemical smog periods) with variable toxicities must be envisaged. The toxicity for individual plants or crops of these mixtures depends on

1. The concentration of the single component within individual plant toxicity thresholds
2. The ratio of concentrations of these compounds
3. The periods of emission of the individual components, i.e., whether they are emitted simultaneously, sequentially, or intermittently. This variability makes prediction of effects of these steadily varying gas mixtures almost impossible. Because of a wealth of careful chamber experiments and field observations during the 1970s and 1980s, certain issues have become substantial.

### 1. SO<sub>2</sub> and Ozone

Close to large cities, combinations of high concentrations of both SO<sub>2</sub> and ozone may occur, although these trace gases stem from completely different sources and reactions. The combined subthreshold emission of 0.1 µl/l leads to a reduction of productivity of several plants in which ozone symptoms appear relatively late and SO<sub>2</sub> not at all. Dependent on the individual species and the time of emission and dose, additive, lower than additive, and overadditive (cooperative) effects are observed; predictions are barely possible. SO<sub>2</sub> and ozone combinations induce a decrease in stomatal conductivity. As far as the photosynthetic capacity is concerned, a cooperative inhibition has been reported for sugar maple (*Acer saccharum*), black oak (*Quercus velutina*), bean (*Vicia faba*), American ash (*Fraxinus americana*), and soybean (*Glycine max*). Generally potentiating damage has to be envisaged in terms of this combination under conditions in which exposure to single-gas doses causes no acute injury. In these cases the single gases are not yet toxins since they can be metabolized individually. The combination no longer allows this metabolism in the sense of detoxification.

### 2. SO<sub>2</sub> and NO<sub>2</sub>

In the proximity of power plants (of the old type!) and low air movement high concentrations of both SO<sub>2</sub> and NO<sub>2</sub> may accumulate. This combination (0.75 µl/l and 1 µl/l, respectively), for example, caused an over-additive effect, whereas treatments with the individual gases had no



influence. The observed symptoms surprisingly showed no similarities to the symptoms of one of the individual gases ( $\text{SO}_2$  or  $\text{NO}_2$ ) but rather the characteristics observed on the leaves after ozone exposure. Thus we can assume that the potentiating effect of the gas combinations is due to the condition that detoxification is achievable at subthreshold individual exposures but no longer effective at combined exposures. In the case of  $\text{SO}_2$  or  $\text{NO}_2$  this assumption is feasible in the sense that the competition for reductive metabolism (see Sec. IV) by photosynthetic electron transport limits the velocity for the reductive incorporation of the individual compounds, thus provoking interfering radical reactions.

### **E. Air Pollution and Freezing**

Low temperatures in winter together with relatively high irradiation and frost dryness have special impact on plants. Evergreen plants such as conifers counteract frost dryness with stomatal closure, composition of their thylakoid lipids (increase of unsaturated fatty acids), increase of proteins and sugars, and increase of the antioxidant enzymes.

It has been observed, however, that frost resistance was decreased by air pollution as a result of several physiological changes such as reduction of the proline content as a frost protectant. In addition the impairment of the cuticular integrity by the combination of frost and acidic deposition may be responsible for electrolyte leakage. This cuticular weathering has been taken as one parameter of the dieback of pine trees in higher altitudes. An increased sensitivity to fungal pathogens due to the loss of structural resistance has also been taken into account.

### **F. Air Pollution and Biogenic Diseases (Infections)**

Since air pollutants have such fundamental impacts on general cellular metabolism it is not surprising that host–pathogen interactions are also influenced by them: since these trace gases act as “antibiotics” (bactericidal, fungicidal, viricidal) it is not surprising that controversial effects are observed in these interactions, so that air pollutants can support as well as counteract biogenic impacts to higher plants

The bactericidal and fungicidal properties of  $\text{SO}_2$  have been in use for centuries for the sterilization of beer and wine barrels; HF treatment was in use in the 19th century for wood preservation and ozone treatment of fruits at concentrations of 1000 and 2000  $\mu\text{g}/\text{m}^3$  as means of sterilization before storage was tried in the 20th century but has not proved very useful and is thus rarely used today.

Support of biogenic diseases is indeed obvious in cases in which

1. Necroses on plant surfaces after acute trace gas exposures allow the entrance of microorganisms (bacteria, virus) that under normal conditions have no chance of penetration or
2. Defense mechanisms such as formation of lignin or callose barriers or phytoalexin formation are weakened as a result of inhibition of energy metabolism (photosynthesis) after chronic gas exposures.

Since ozone is the trace gas of greatest relevance to both human and plant health in our atmosphere the effects of ozone exposure and the mechanisms underlying its influences on plant metabolism (memory effect; and systemic acquired resistance [SAR]) are discussed in Chapter 11.

#### IV. DETOXIFICATION

We generally have to envisage two basic possibilities of how plants deal with air pollutants:

1. The trace gas is known to the plant since it has already been present during evolution
2. The trace gas is xenobiotic to the plant, meaning the plant has had no chance to adapt during evolution.

There is good evidence that plant metabolism is generally very well adapted (however, with large individual differences!) to relatively high fluxes of  $\text{SO}_2$ ,  $\text{NO}_2$ , and ozone. There exist several possibilities either to metabolize these gases in the sense of substrates or even of nutrients ( $\text{SO}_2$ ,  $\text{NO}_2$ ) or to counteract their damaging effects.

The following reactions contribute to these detoxifying and repair processes:

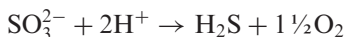
1. Small molecules such as ascorbate, tocopherols, glutathione, diphenols, or carotenoids act as antioxidants and scavengers of free radicals produced during the process of interaction of the corresponding air pollutant with a vitally important cellular component
2. Enzymes such as DNA or RNA restriction enzymes, nucleases, polymerases, peroxidases, catalase, superoxide dismutases, and reductases, detoxify problematic intermediates or repair developing or ongoing damage
3. Metabolic sequences such as the ascorbate peroxidase–glutathione reductase pathway or anaplerotic sequences (that complete deficient steps in metabolism) are initiated

4. Hormone-dependent corrections and alterations of the direction of metabolic events occur, for example from catabolic to anabolic pathways.

Via these different (and perhaps many other) mechanisms plants are able to tolerate and to adapt quickly to sometimes extreme concentration of these air pollutants.

### A. SO<sub>2</sub>

SO<sub>2</sub> penetrates the plasmalemma and is converted very quickly, mainly in the chloroplast, into SO<sub>3</sub><sup>2-</sup>, which is either converted to sulfate or (photosynthetically) reduced to hydrogen sulfide:



Sulfide in turn can be utilized to form sulfur-containing amino acids such as cysteine, methionine, or glutathione. Plant proteins contain only c. 1% sulfur. Thus, fast growing, protein-rich herbaceous plants prove more tolerant to sulfur dioxide exposures than trees, which contain only a small percentage of protein.

### B. NO<sub>2</sub>

NO<sub>2</sub>, similarly to SO<sub>2</sub>, readily penetrates plant cuticles and plasmalemma, yielding nitrous and nitric acid in the intracellular aqueous phase. In the mesophyll both nitrite and nitrate are reduced to ammonia ions in a light-dependent manner:



Since plant proteins contain approximately 16% nitrogen, the demand for reduced nitrogen in the plant cell is high. As a result atmospheric NO<sub>2</sub> must be seen rather as a fertilizer than as a toxin.

### C. Ozone

Ozone in contact with the aqueous layer on membranes quickly decomposes, and its concentration decreases with increasing distance from the gaseous phase. Most ozone is thus trapped in the plasmalemma. This is the reason why membranes of cell organelles are scarcely damaged. Unsaturated fatty acids in the plasmalemma undergo ozonizations (i.e., endogenous peroxidations; see earlier discussion), yielding peroxides, which in turn are

reductively metabolized either by microsomal electron transport systems or by the glutathione system (see later discussion). In contrast to exposure to NO<sub>2</sub> or SO<sub>2</sub>, ozone treatment does not result in the accumulation of a characteristic element.

### 1. Detoxification of Highly Reactive Intermediates

Reactive oxygen intermediates such as superoxide, hydrogen peroxide, singlet oxygen, and other organic peroxidic or radical intermediates stemming from interactions of oxidative stress (see Chapter 2) are detoxified in the stroma of the chloroplast, where ascorbic acid plays a pivotal role.

Ascorbate reacts spontaneously with superoxide, forming dehydroascorbate and hydrogen peroxide. In contrast, detoxification of H<sub>2</sub>O<sub>2</sub> via ascorbate (AA) is catalyzed by the enzyme ascorbate peroxidase (AA-POD). The regeneration of dehydroascorbate back into the reduced form is driven by reduced glutathione (GSH), which in turn is regenerated at the expense of NADPH via glutathione reductase (GR).

Another detoxification sequence also involving GSH is the conversion of organic acyl hydroperoxides (ROOH) into the corresponding alcohols catalyzed by glutathione-S-transferase (GST) in this case operating as a peroxidase:



The oxidized glutathione, GSSG is reduced back to GSH via glutathione reductase (GR) catalysis, as discussed.

Another detoxifying pathway also including ascorbate (AA) and, in addition monodehydroascorbate (MDHA), operates partially via tocopherol (Toc), again at the expense of NADPH. In this pathway the extremely reactive species singlet oxygen and OH radical may react primarily with carotenes or fatty acids (RH). The emanating free radicals (R•) are directly repaired by Toc or indirectly by the one-electron redox pair, AA–MDHA.

It has been shown that during exposure to ozone or sulfur dioxide the ascorbate and glutathione concentrations are increased in several plants (mung beans, peas, pine needles); in other cases a reduction has been found. An induction of enzymes involved in the detoxification (SOD, GR, AA-POD, MDHA-R) has been reported for herbaceous plants as well as for trees. Some of these inductive effects on enzymes (AA-POD) could be achieved (in beans and peas) by ethene pretreatment, resulting in decreased sensitivity to ozone.

## V. PLANT–CLIMATE INTERACTIONS: EMISSION AND REACTIONS OF BIOGENIC VOLATILE ORGANIC COMPOUNDS AND NO<sub>x</sub>

Biogenic volatile organic compounds (BVOCs) emitted into the atmosphere may constitute up to 10% of the carbon fixed by photosynthetic processes of individual plants. As reviewed by Penuelas and Llusia (46), the total global emission rate may rise to 10<sup>9</sup> t/y, representing approximately 80% of the reactive VOCs in the atmosphere. Thus, only 20% of this VOC contribution is anthropogenic, according to this calculation. These VOCs may have a dual activity: short-term function as bioregulators and long-term function as climatic effectors with impacts on climate warming, in which both positive as well as negative impacts have to be considered.

Volatile terpenoids and other classes of organic compounds such as acids, aldehydes, esters, alkanes, and alkenes (to name just a few) have diverse biological functions and activities. Monoterpene emissions, as one class of the most important volatile organic compounds (VOCs), have been intensively studied in the last couple of years, and interesting defense and allelopathic functions (see Chapter 2) have been reported. These functions include vital properties such as deterrence against herbivores, wound sealing, communication in an allelopathic sense, and attraction of herbivore predators and pollinators. Most important and directly connected to climatic affairs is thermotolerance, another effect associated with VOC emission. Volatile terpenes not only have beneficial impacts on plants but have long been used as herbal medicinal products for the treatment of gastrointestinal diseases, pain, cold, and bronchitis, with proven bioavailabilities (47).

Both internal (genetic and biochemical) as well as external factors are basically responsible for production and release of these emanations. These external factors comprise biotic (animal, plant, and microbe interactions) as well as abiotic factors, which in turn may directly represent a feedback of climate, such as relative humidity and water availability, light quality and intensity, temperature, wind, and ozone. In extremely different individual ways, these factors may govern the biosynthesis of BVOCs, which mainly are not spontaneously released but are internally stored in specific cellular compartments or small temporary pools. From these different pools BVOCs are slowly released, depending on the specific external factors and finally evoke different biological (see earlier discussion) and environmental effects. These effects concern atmospheric chemistry (CO production, methane lifetime, ozone dynamics) as well as positive and negative feedbacks on climate, e.g., warming. These different emissions have been

**Table 11** Estimated Annual Global Emission of Biogenic Volatile Organic Compounds (as  $10^6$  Tons Carbon)

Isoprenes	Monoterpenes	Other BVOCs <sup>a</sup>	Ethene
175–503	127–480	ca. 520	1–20

<sup>a</sup>BVOCs, biogenic volatile organic compounds.

Source: Ref. 46.

roughly calculated in terms of the percentage contribution of the main VOC groups (Table 11).

Emission of BVOCs may be extremely variable for different species at different sites, conditions, and times, ranging from 0 to 100  $\mu\text{g/g}$  dry matter/h, comprising up to 8%–10% fixed carbon (see earlier discussion).

As mentioned an important new feature in the production and function of monoterpene emission is that induction of thermotolerance may be a major effect, and coupling of this process to photorespiration seems to be indicated, although not at all understood. This may imply that only C-3 and not C-4 plants essentially contribute to these emissions.

Recent global changes due to altered land use,  $\text{CO}_2$  level increase, and increased N emissions (traffic; agriculture) may thus enhance the greenhouse effect not only directly but also indirectly via the increase of BVOC emissions and, according to certain scenarios, also climate warming. Climate warming in turn may increase photorespiration and thus in turn release of BVOCs. According to certain feedback models, BVOC-derived aerosols exhibit a retarding effect on climate warming, whereas release of latent heat and water condensation have an enhancing effect. Either directly or indirectly through increase of ozone or methane lifetime BVOCs increase the greenhouse effect.

In this context a 2003 finding might be of extreme importance: UV-induced release of  $\text{NO}_x$  from plants. As reported in 2003 for Scots pine experimentally held in quartz chambers (48), plants may not only act as sinks for nitrogen oxides (see Sec. IV) but also emit  $\text{NO}_x$  under UV influence. Since uptake and emission occur simultaneously, the compensation point has been calculated as 1 ppb for cloudy days at the experimental station in southern Finland. At maximal UV irradiance at this station this point has been calculated to rise well above 3 ppb. This compensation point was formerly strongly underestimated since all previous experiments were not performed in (extremely expensive!) quartz chambers, but in containers made of material excluding UV irradiation.

The emission rate of  $\text{NO}_x$  was measured as  $1 \text{ ng/m}^2$  needle surface per second under conditions of 0.5 ppb ambient  $\text{NO}_x$ , a value reflecting preindustrial conditions.

The authors conclude that global  $\text{NO}_x$  emissions from the boreal area may be “comparable to those produced by worldwide industrial and traffic sources”.

Together with the emission of BVOCs,  $\text{NO}_x$  emission from plants according to these new data may considerably contribute to conditions that govern climate-relevant parameters and thus ecology in its widest sense.

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# 5

## Limitation of Salt Stress to Plant Growth

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### I. INTRODUCTION

#### A. Importance of Salinity in Agriculture

Salinity in soils affects about 7% of the world's total land area (1). Of the cultivated land, 23% is saline. Furthermore, about 17% of the world's cropland is under irrigation, but irrigated agriculture contributes well over 30% of the total agricultural production (2). More importantly, secondary salinization in irrigated lands is of major concern for global food production as well. Currently, about 20% of irrigated land in world average has suffered from secondary salinization and 50% of irrigation schemes are affected. There is also a dangerous trend of a 10% per year increase in the saline area throughout the world (3). In addition, salinity is a problem for agriculture because few crop species are adapted to saline conditions.

#### B. Definition of Salinity

*Salinity* as defined herein is the concentration of dissolved mineral salts present in soils (soil solution) and waters. The dissolved mineral salts consist of the electrolytes of cations and anions. The major cations in saline soil solutions consist of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{K}^+$ , and the major anions are  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ , and  $\text{NO}_3^-$ . Other constituents contributing to salinity in hypersaline soils and waters include B,  $\text{Sr}^{2+}$ ,  $\text{SiO}_2$ , Mo,  $\text{Ba}^{2+}$ , and  $\text{Al}^{3+}$ .

These salinity constituents are reported in units of millimoles per liter (mmol/l) or millimoles charge per liter (mmol charge/l) (meq/l) or milligrams per liter (mg/l) (parts per million [ppm]). The parameters often used to evaluate salinity in soils are electrical conductivity (EC), total dissolved solids (TDS), and osmotic potential ( $\Psi_{\pi}$ ).

The relations between these parameters are as follows:

$$\Psi_{\pi} \text{ (MPa)} \cong -0.036 \times \text{EC (dS/m)}$$

$$\text{TDS (mg/l)} \cong 640 \times \text{EC (dS/m)}$$

On the basis of soil EC of the extract of a saturated soil paste ( $\text{EC}_e$ ), sodium ion percentage of soil cation exchange capacity (ESP), and pH of saturated soil paste (pHs), saline soils can be defined as  $\text{EC}_e > 4$  dS/m,  $\text{ESP} < 15\%$ , and  $\text{pH} < 8.5$  (4).

However, this salinity criterion is a relative one because there is substantial difference in salt tolerance among plants.

### C. Impacts of Salinity

Salinity not only decreases the agricultural production of most crops, but also, as a result of its effect on soil physicochemical properties, adversely affects the associated ecological balance of the area.

The following are some of the harmful impacts of salinity:

- Low agricultural production

- Low economic returns due to high cost of cultivation, reclamation, management, etc.

- Soil erosion due to high dispersibility of soil

- Ecological imbalance due to a change in plant cover from glycophytes to halophytes and marine life forms from fresh water to brackish water

- Poor human health due to toxic effect of elements such as B, F, and Se.

### D. Causes of Salinity

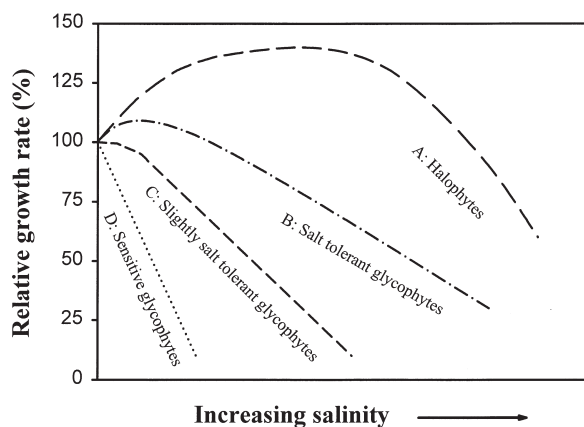
In principle, elevated salinity level in soils results mainly from two sources: natural and man-made. Salinity in arid and semiarid areas is mainly caused by natural causes, i.e., low precipitation, high level of evaporation, existence of saline parent rock, and hydrological conditions (5). However, salinity also results from mismanaged amelioration systems, poor technique of irrigation, irrigation with salinized water, salt accumulation from high doses of mineral fertilization (5), grazing, and deforestation.

### E. Plant Growth Response to Salinity

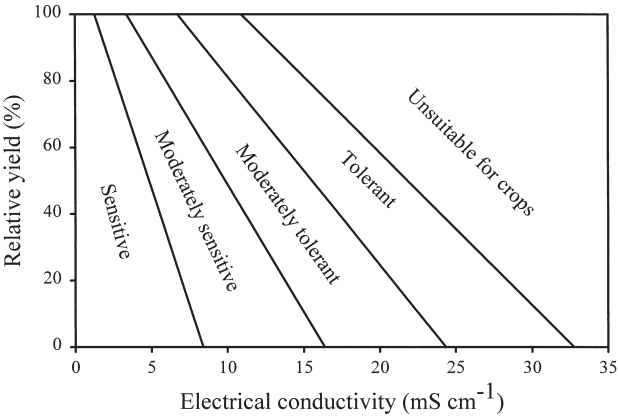
There is the diversity in salt tolerances between species. In general, the term *halophytes* refers to salt-tolerant plant species. The growth of halophytes (Fig. 1, line A) is optimal at relatively high level of salinity and they are capable of accumulating relatively high quantities of salt in their tissues as mineral nutrients. Most crop plants are nonhalophytes, i.e., glycophytes. Only a few crop species are slightly stimulated by low salinity levels (Fig. 1, line B). The salt tolerance of glycophytes is relatively low (Fig. 1, line C) or their growth is severely inhibited even at low substrate salinity levels (Fig. 1, line D).

Salt tolerance is usually assessed by physiologists as the percentage biomass production in saline versus control conditions over a prolonged period. Dramatic differences are found between plant species. As illustrated in a review by Greenway and Munns (6), after some time in 200 mM NaCl, a salt-tolerant species such as sugar beet might have a reduction of only 20% in dry weight, a moderately tolerant species such as cotton might have a 60% reduction, and a sensitive species such as soybean might be dead.

*Crop salt tolerance* can be defined as the ability of plants to survive and produce economic yields under the adverse conditions caused by soil salinity. Salt tolerance of agricultural crops is typically expressed in terms of yield decrease associated with soil salinity increase or as relative crop yield on saline versus nonsaline soils (7). Generally, classification of the salt tolerance (or sensitivity) of crop species, forage species, and fruit trees is based on two parameters: the threshold EC and the slope, i.e., percentage of



**Figure 1** Schematic graph indicating the growth response of halophytes and glycophytes to salinity.



**Figure 2** Divisions for crop salinity tolerance classification based on the relationship between relative crop yield and salinity (expressed in terms of electrical conductivity at 25°C). (Adapted from Ref. 7.)

**Table 1** Tolerance of Crop Species to Soil Salinity

Crop species	EC saturation soil extract (EC <sub>e</sub> )		Tolerance rating
	Threshold, <sup>a</sup> dS m <sup>-1</sup>	Slope, <sup>b</sup> % per dS m <sup>-1</sup>	
Barley ( <i>Hordeum vulgare</i> )	8.0	5.0	Tolerant
Sugar beet ( <i>Beta vulgaris</i> )	7.0	5.9	Tolerant
Bermuda grass ( <i>Cynodon dactylon</i> )	6.9	6.4	Tolerant
Wheat ( <i>Triticum aestivum</i> )	6.0	7.1	Moderately tolerant
Tomato ( <i>Lycopersicon esculentum</i> )	2.5	9.9	Moderately tolerant
Maize ( <i>Zea mays</i> )	1.7	12.9	Moderately tolerant
Orange ( <i>Citrus sinensis</i> )	1.7	16.0	Sensitive
Grapevine ( <i>Vitis</i> sp.)	1.5	22.0	Sensitive
Bean ( <i>Phaseolus vulgaris</i> )	1.0	19.0	Sensitive

<sup>a</sup>Threshold EC<sub>e</sub> (25°C), Maximal soil salinity that does not reduce yield.

<sup>b</sup>Slope, yield reduction per unit increase in EC<sub>e</sub> beyond threshold.

EC<sub>e</sub>, Electrical conductivity of soil saturation extract.

Source: Adapted from Ref. 8.

yield decrease beyond the threshold. Examples taken from an extensive study area given in Fig. 2 and Table 1. It is evident that barley tolerates relatively high salinity levels in comparison, for example, with bean or grapevine.

There are differences in salt tolerance between cultivars within a crop species. The genetic variability within a species is not only a valuable tool for studying mechanisms of salt tolerance, but also an important basis for screening and breeding for higher salt tolerance.

Plant organs and growth stages may respond to salinity differently, depending on the plant species, cultivars, or environmental factors. Sugar beet, for example, is highly tolerant during most of its life cycle but sensitive during germination. In contrast, the salt sensitivity of rice, tomato, wheat, and barley usually is higher in vegetative stage than in germination (7). In general, the leaf growth is most sensitive to salinity. For example, limitation of salinity to wheat growth may be mainly due to the reduction in leaf growth, as is true also for other grass species (9, 10). A general pattern in the plant response to salinity is that the larger the change in the root/shoot ratio, the greater the effect of salinity on the productivity (11).

## **F. Solutions for Salinity**

Possible solutions can generally be summarized as (a) restriction of salinization by leaching of salts from the root zone, (b) cropping management, and (c) use of tolerant plants.

### **1. Leaching of Salts from Root Zone**

Soil scientists have devised many reclamation methods and management practices to reduce salt stress (4). For reclamation of saline soils, leaching of surface salts has been widely recommended because of normal permeability of those soils; the salts are usually leached below the root zone whenever the amount of water infiltrated exceeds that lost by evapotranspiration. In contrast, in arid and semiarid regions where rainfall is low and irrigation water is saline, it is difficult to achieve adequate leaching (12).

### **2. Irrigation Techniques**

Salinization of irrigated agriculture can be prevented by better irrigation practices such as adoption of partial root zone drying methodology and drip or microjet irrigation to optimize use of water.

### **3. Nutrient Management**

Salinity causes nutrient imbalance, resulting from the effect of nutrient availability in growth medium or an increase in the requirement of the plant for essential elements (e.g., N, K<sup>+</sup>, and Ca<sup>2+</sup>). Nutrient management should take account of the following (a) under low salt stress, nutrient deficiency

limits plant growth to a greater extent than salinity; a positive interaction or increased salt tolerance results; (b) under moderate salinity, nutrient deficiency and salinity may equally limit plant growth, and no interaction occurs; and (c) under high salinity, salinity limits growth to a greater extent than nutrient deficiency.

#### 4. Cropping Management

Farming systems can change to incorporate perennials in rotation with annual crops (phase farming), in mixed plantings (alley farming, intercropping), or in site-specific planting (precision farming). In precision farming, areas of high production can also be identified, and these sites can be planted with cultivars of high vigor that use water effectively during the growing season and consume most of the available soil water. Phase farming, in which several years of pasture are rotated with several years of crop, can make use of deep-rooted pasture plants to dry the deep subsoil, thereby creating a buffer zone to hold any water that escapes the crops (13).

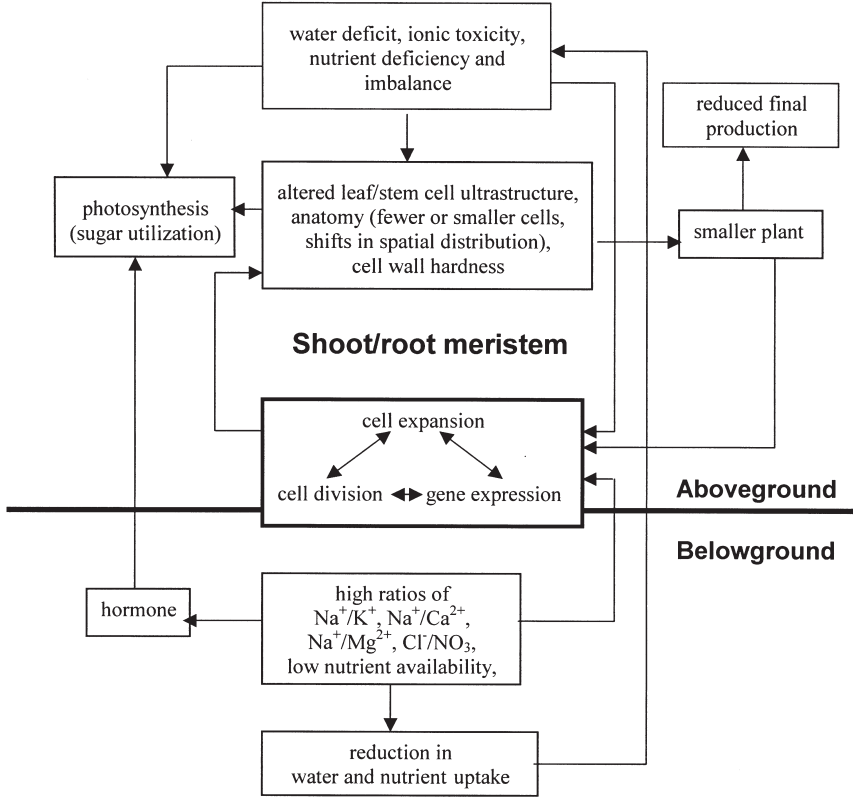
#### 5. Salt-Tolerant Crops

Although the use of some management options can ameliorate yield reduction under salinity stress, implementation is often limited because of cost and availability of good water quality or water resource. Using the salt-tolerant crops is one of the most important strategies to solve the salinity problem. To increase the plant salt tolerance, there is a need for understanding of the mechanisms of salt limitation on plant growth and the mechanism of salt tolerance at the whole-plant, organelle, and molecular levels.

## II. LIMITATION OF SALINITY TO PLANT GROWTH

Under saline conditions, soils contain extreme ratios of  $\text{Na}^+/\text{Ca}^{2+}$ ,  $\text{Na}^+/\text{K}^+$ ,  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , and  $\text{Cl}^-/\text{NO}_3^-$ . The growth inhibition due to salinity may be caused primarily by the osmotic stress, ionic effect, and ionic imbalance, acting on biophysical and/or metabolic components of expansive growth (14). These components may be described more elaborately as (a) the average decrease in soil water potential as a function of time and effective root zone area; (b) the toxic effects of ions as a function of time, salt concentration, and composition; and (c) the limitation of nutrients as a function of specific ratios between nutrients and competitive ions (i.e.,  $\text{Na}^+/\text{K}^+$ ,  $\text{Na}^+/\text{Ca}^{2+}$ ,  $\text{Cl}^-/\text{NO}_3^-$ ) (Fig. 3).

A conceptual model illustrating the relations among salinity effects (osmotic, ion toxicity, and ionic imbalance) and transduction (assimilate



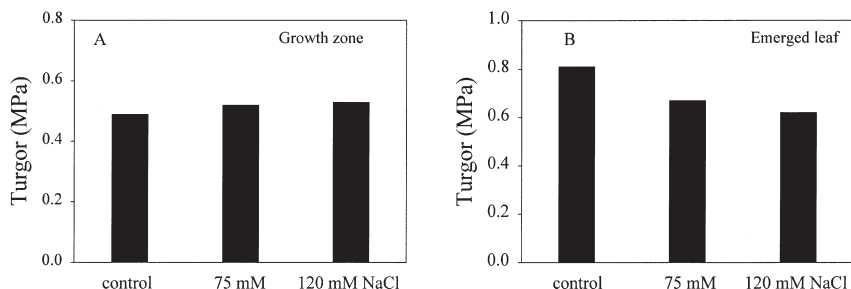
**Figure 3** Conceptual model indicating the limitation of salinity to plant growth.

partitioning, root-to-shoot signaling, differential gene expression, hormones) and adaptation (morphological, anatomical, and ultrastructural) is presented in Fig. 3. Growth responses to salinity may be directly due to osmotic and ionic effects and indirectly due to photosynthesis or chemical and/or biochemical messengers. In this section, we attempt to understand how plant growth is affected by increasing salinity by integrating what is known at the levels from molecular and organelle to whole plant.

**A. Osmotic Effects**

Under saline conditions, low osmotic potentials of the soil solution induce water deficit in plant tissue. As a consequence, cell turgor pressure decreases. Since the growth of cells is correlated with turgor pressure in the growing tissues, decreased turgor is the major cause of inhibition of





**Figure 4** Epidermal cell turgor pressure of barley leaf grown under control conditions or in nutrient solution containing 75 and 120 mM NaCl: A, in the growth zone; B, in the emerged part. (Modified from Ref. 19.)

plant cell expansion under saline conditions (6). However, Munns (15) suggested that turgor is unlikely to play a role in the reduction of cell elongation in the growing leaf under saline conditions. There are only a few measurements of turgor in growing tissues of salt-affected plants. Thiel and associates (16) and Yeo and colleagues (17) using the pressure probe, found no detectable change in the turgor of elongating cells of plants grown in saline solution. According to the 1993 studies for wheat by Arif and Tomos (18) and 2002 studies for barley by Fricke and Peters (19), the turgor at the cell level measured with the pressure probe showed no clear relation to leaf elongation in the growth zone under saline conditions (Fig. 4A). That is, either turgor in elongating tissues is not affected by water deficit (e.g., Refs. 20 and 21) or, when it is, there is no correlation between the local elongating rate and the turgor of the cells (e.g., Refs. 22 and 23). In the emerged (air-exposed) leaf blade, i.e., maturity tissues, turgor was always higher than in actively growing cells and decreased significantly with increasing salt stress (Fig. 4B) (19).

Lack of change in turgor of the growing tissues under saline conditions may be due to osmotic adjustment, which helps to maintain the turgor in cells. Elongating cells adjusted osmotically to changes in external water potential by accumulating more solutes and by reducing the volume expansion (19, 24). Fricke and Peters (19) suggested that at high salinity levels, the largest increase in osmolality is achieved by reduced volume expansion rather than by increased solute deposition rates, a finding that can be explained in two ways: First, the total amount of solutes available for osmotic adjustment may have been limited, already reaching maximal deposition rates at moderate stress level. Sugar and other organic solutes contribute little to osmolality along the growth zone of NaCl-stressed wheat leaves (24). Therefore, the rate at which inorganic solutes

were supplied to the growth zone may have limited cell expansion. As a consequence, the rate of cell expansion had to slow to allow maintenance of the gradients of water potential between elongating cells and the xylem solution.

Second, a limitation in the rate at which solutes were taken up and deposited may have caused cells at high salinity (e.g., 120 mM NaCl) to grow most slowly. It is possible that expanding cells of plants exposed to high salinity levels were metabolically or energetically limited in their ability to accumulate solutes at rates as high as those of plants exposed to moderate salinity (25). If so, cells needed to expand and dilute solute contents at lower rates to maintain the gradients of water potential and water uptake.

## B. Ionic Effect

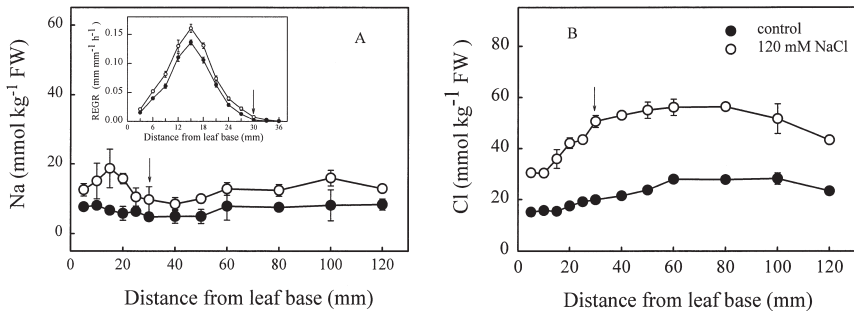
Considerable attention has been focused on the hypothesis that  $\text{Na}^+$  or  $\text{Cl}^-$  may be toxic to plants. In support of the hypothesis, the positive correlations between salt tolerance and  $\text{Na}^+$  exclusion have been shown by Drew and Läuchli (26), Schubert and Läuchli (27), and Lazof and Bernstein (28). Accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in the leaves, through the transpiration flow, is generally a long-term process occurring in salt-stressed plants (29). High internal concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  may provide toxic ions in the cellular compartment (6, 30, 31).

Plant growth is affected by the interactions of  $\text{Na}^+$  or  $\text{Cl}^-$  and many mineral nutrients, causing imbalances in the nutrient availability, uptake, or distribution within plants and also increasing the plant's requirements for essential elements (6, 14, 32). For example, high concentrations of  $\text{Na}^+$  in the external solution caused decreases in  $\text{K}^+$  and  $\text{Ca}^{2+}$  concentrations in the tissues of many plant species (6, 32, 33). The decrease may be due to the antagonism between  $\text{Na}^+$  and  $\text{K}^+$  or  $\text{Ca}^{2+}$  at sites of uptake in roots, to the effect of  $\text{Na}^+$  on the  $\text{K}^+$  and  $\text{Ca}^{2+}$  transport into the xylem (32, 34, 35), or to indirect inhibition of the uptake process in other aspects, for example, hydrogen-adenosine triphosphatase ( $\text{H}^+$ -ATPase) activity (36, 37). Since  $\text{Ca}^{2+}$  is essential for maintaining selectivity and integrity of cell membranes (38, 39), the deficiency of  $\text{Ca}^{2+}$  could impair both the selectivity and the integrity of the membrane and then accelerate the passive accumulation of  $\text{Na}^+$  in plant tissues.

However, much of the physiological research into salinity has concentrated on ionic effects (ionic toxicity and nutritional disturbance) in whole plants or in nongrowing tissues. The measurements in whole plants or nongrowing tissues do not allow estimation of the correlations between growth and direct causes. Leaf elongation in grasses, i.e., the growth zone, is restricted to a small region at the base of the blade enclosed by older

leaves (40). Although the growth zone is enclosed, grass leaves present a good opportunity to study leaf growth processes, because the growth zone is distinct and relatively simply organized (41). Elongation is largely unidirectional, and a cellular particle is displaced away from the leaf base as a result of the production of younger tissue and longitudinal growth. Since the growing tissues are most active in metabolism and strong sinks for nutrients, the leaf growth of grasses under control or stress conditions should be much more closely associated with metabolic and nutritional changes within the most actively growing tissues than within the whole plant or nongrowing tissues. By comparing the profiles of spatial distribution of leaf elongation of grasses with that of mineral elements, sugars, and water relations in growing tissues of grasses with and without salinity, it is possible to determine the causes of the direct effect of salinity on leaf elongation of grasses.

The results of  $\text{Na}^+$  elemental analyses performed on the same scale as the growth analysis for the growing leaves of sorghum and wheat rule out sodium toxicity as a direct cause of growth inhibition (Fig. 5A) (42). Although Fig. 5A showed that  $\text{Na}^+$  concentration was much higher at salt treatment than at control, the level of  $\text{Na}^+$  concentration is far below the level of ionic toxicity in the leaf tissues (42). This finding supports suggestions (43) that the leaf growth of barley is not directly controlled by the local concentration of  $\text{Na}^+$  in growing tissues. Furthermore, the pattern of spatial distribution of  $\text{Na}^+$  in the leaf growth zone of wheat under saline conditions



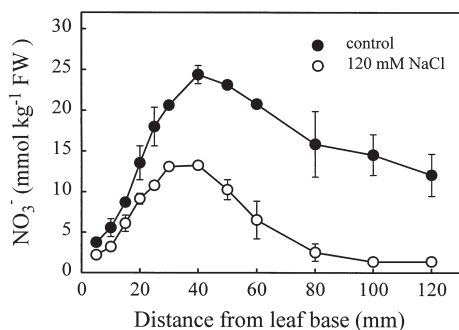
**Figure 5** Sodium (A) and  $\text{Cl}^-$  (B) concentrations along the growth and maturity zones of wheat leaf 4 grown in soil with no added NaCl and 120 mM NaCl. Error bars ( $n = 2$ ) represent standard errors and fit within the plot symbol if not otherwise shown. Arrow indicates the position of the end of the leaf growth zone. Inset illustrates the relative elemental growth rate (REGR) along the growth zone of wheat leaf 4 grown in soil with no added NaCl and 120 mM NaCl: FW, Fresh weight. (Modified from Ref. 42.)

was closely related to that of the relative elongation growth rate of wheat leaf along the leaf axis (Fig. 5A, inset), a relationship that is contrary to the behavior expected from growth inhibition. Along the leaf base there is a gradient in vacuolization. As cells elongate, during their displacement from the leaf base, larger vacuoles develop. It is unlikely that the degree of tissue vacuolization regulates  $\text{Na}^+$  accumulation in the tissue, since the pattern of increased accumulation does not coincide with vacuole volume.

Hu and Schmidhalter (42) suggested that although  $\text{Cl}^-$  level in the growth zone of wheat under saline conditions was about four times higher than  $\text{Na}^+$  level (Fig. 5b), it was also unlikely to be toxic. The maximal  $\text{Cl}^-$  content at the end of the elongating zone reached only (50–60  $\text{mmol kg}^{-1}$  fresh weight [FW]) (Fig. 5b), and this concentration did not inhibit in vitro protein synthesis in a wheat germ system (44). In an earlier study by Hu and colleagues (9),  $\text{Cl}^-$  concentration in the mature leaves of wheat was about 10 times as high as that in growing leaves under similar conditions and had very little effect on the main stem grain yield. These results together suggest that the growing leaves may be able to regulate the  $\text{Cl}^-$  concentration to prevent an excessive accumulation of ions, because the expanding vacuoles in growing tissues would readily accommodate the salts and so prevent their buildup in the cytoplasm or the cell wall. Munns and coworkers (43, 45) found that  $\text{Cl}^-$  concentration in the growth zone of barley under saline conditions is unlikely to cause toxicity to leaf elongation as well. Chloride is very mobile, and plants tolerate it at high concentration (46). It preferentially accumulates in the old leaves and in the leaf sheath (6, 47).

For interaction of  $\text{Na}^+$  or  $\text{Cl}^-$  and other nutrient elements, the direct elemental analyses in the growing leaves of wheat at 120 mM NaCl (42) showed that  $\text{K}^+$  and  $\text{Ca}^{2+}$  concentrations were increased by salinity, especially in the elongation zone; this finding contrasted with findings of studies on growing leaves of sorghum (48) and on maize roots (49). This contrast was most likely due to the high  $\text{Ca}^{2+}$  content in the soil studied by Hu and Schmidhalter (42). Calcium enhanced the uptake of  $\text{K}^+$  in pigeon pea (*Cajanus cajan* L. Huth), resulting in a higher concentration of  $\text{K}^+$  in plants grown under saline conditions (50). Cramer and associates (51) reported higher concentrations of  $\text{K}^+$  in the root tips of two corn cultivars in the presence of calcium. Similarly, higher external  $\text{Ca}^{2+}$  level may cause a higher  $\text{Ca}^{2+}$  concentration under saline conditions. Nevertheless,  $\text{K}^+$  and  $\text{Ca}^{2+}$  contents in the leaf tissue are unlikely to limit leaf elongation of wheat under saline conditions. With these points in mind, however, the inconsistent data demonstrate that the effect of salinity on  $\text{K}^+$  and  $\text{Ca}^{2+}$  in young tissue is different for various plant species and growth media.

Barnal and colleagues (52) proposed that the relatively greater uptake of  $\text{Cl}^-$  than of  $\text{Na}^+$  in salt-stressed plants may also be responsible



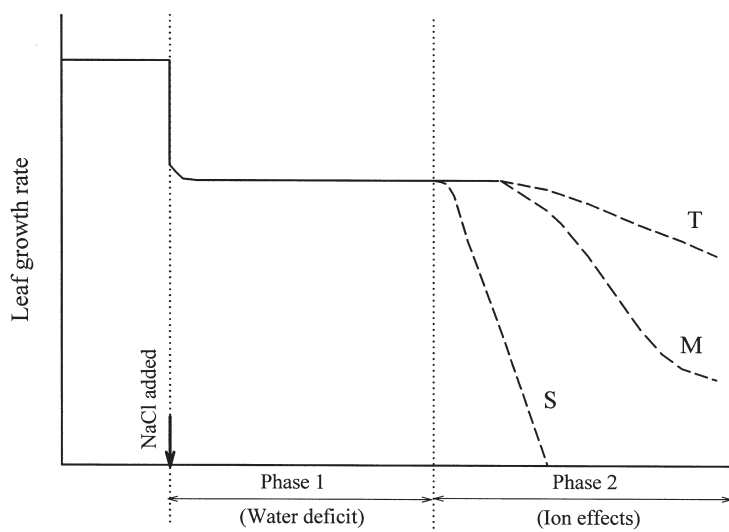
**Figure 6** Nitrate concentration along the growth and maturity zones of wheat leaf 4 grown in soil with no added NaCl and 120 mM NaCl. Error bars ( $n=2$ ) represent standard errors and fit within the plot symbol if not otherwise shown. Arrow indicates the position of the end of the leaf growth zone. FW, Fresh weight. (Modified from Ref. 42.)

for the growth reduction by depressing the uptake of other anions such as  $\text{NO}_3^-$ . Although the effect of salinity on  $\text{NO}_3^-$  in the whole plant or mature tissues is well described, much less is known about the effect of salinity on  $\text{NO}_3^-$  in growing tissues. The lower supply of  $\text{NO}_3^-$  to growing leaves may be limiting leaf elongation of wheat by salinity (Fig. 6) (42). The difference in  $\text{NO}_3^-$  concentration between the two treatments increased along the leaf axis. The decrease in  $\text{NO}_3^-$  concentration with distance beyond the elongation zone may be due to the greater reduction of  $\text{NO}_3^-$  in the exposed part of the leaf, since light stimulates the  $\text{NO}_3^-$  reduction, and/or due to the decreased  $\text{NO}_3^-$  uptake by salinity. However, Cram (53) showed that net  $\text{NO}_3^-$  influx was reduced by a high  $\text{Cl}^-$  concentration in root tissue, such that if the abundance of NaCl increased, the concentration of  $\text{NO}_3^-$  decreased.

There are very few data on effects of salinity on micronutrients in growing leaves of grasses. Hu and coworkers (54) reported that salinity affected the distribution pattern of Fe concentration on the FW basis, whereas it did not affect those of Zn and Mn. Therefore, the decreased leaf growth is probably not due to the causes of toxicity or deficiency of these micronutrients in the growing leaves of wheat.

### C. Osmotic and Ion Effects on a Time Scale: Biphasic Model

The two-phase model for the inhibition of growth by salinity was proposed by Munns (15). The first phase of growth reduction in this model is due to



**Figure 7** The two-phase model for salt inhibition of plant growth. T, Tolerant crop; M, moderately tolerant crop; S, sensitive crop to salinity. (Adapted from Ref. 15.)

the osmotic effect of soil salinity (Fig. 7). Early in phase 1 of growth reduction, turgor pressure has an influence on growth, but the majority of phase 1 growth inhibition is maintained and regulated by hormonal signals from roots (time scale here is from hours to weeks). The rate of leaf expansion can similarly be influenced by other osmotica (17, 55, 56), so this effect is extremely unlikely to be salt-specific. Thus, phase 1 of growth reduction depends on salt outside the plant rather than salt in tissues.

During extended periods, i.e., the second phase, salt begins to accumulate in older leaves, and salt injury becomes apparent. The rate of growth reductions in phase 2 depends on the rate of leaf turnover. If a few of these older leaves die and many new leaves are still produced, then the rate of phase 2 growth inhibition is minimized. However, if the number of older leaves affected approaches the rate of new leaf production, then there are changes in the flow of assimilates or hormonal balance, leading to a more rapid phase 2 growth inhibition.

The hypothetical two-phase growth response is illustrated in Fig. 7 by comparing closely related genotypes differing in salt tolerance. The differences in growth response to salinity among varieties of a crop should only be evident during the second phase of inhibition, as was found for wheat, barley, and corn varieties (57, 58). However, in some reports,

variation in salinity responses among genotypes of a species did not correspond to differential phase 2 effects (59–61).

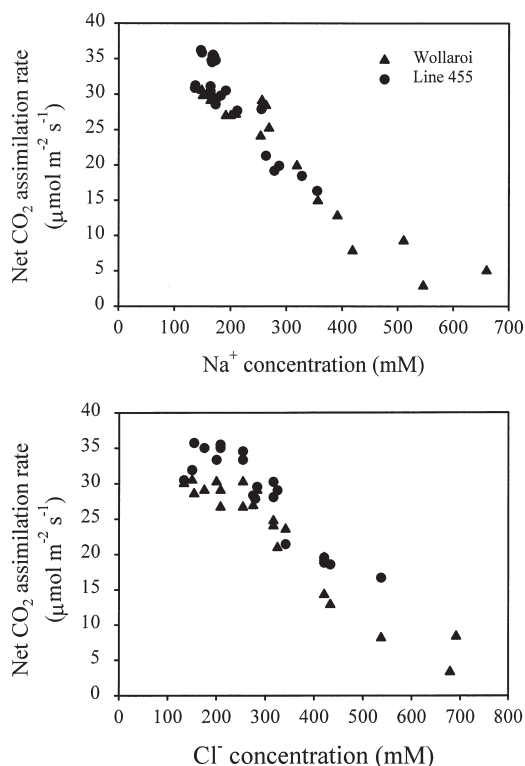
The biphasic model of growth inhibition by salinity describes glycophyte responses to salinity well. However, growth responses to salinity in halophytes do not follow the same model. Halophytes are able to avoid the development of phase 2 growth inhibition by a number of physiological, morphological, anatomical, and behavioral mechanisms.

#### **D. Photosynthesis**

At low or moderate soil salinity, decreased growth is primarily associated with a reduction in photosynthetic area, rather than a reduction in photosynthesis per unit leaf area (15). At high salinity, however, leaf photosynthesis can be reduced by lowered stomatal conductance as a result of water imbalance (62) or by a change in the ionic relations of the chloroplasts (63). In addition, the transport of photosynthates in the phloem may be inhibited (64).

Reductions in photosynthesis due to nonstomatal factors may be caused by toxic ions. Correlations have been observed in a number of species, including bean (65), cotton (66), citrus (67), grapevine (68), and rice (69). Evidence in support of this hypothesis is found in strong negative correlations between ions and photosynthetic activity, in which  $\text{Na}^+$  has been implicated primarily in crop species such as rice (69) and wheat (70), and  $\text{Cl}^-$  in woody perennials such as citrus (71) and grapevine (68, 72). Fig. 8 (73) shows negative relationships between both  $\text{Na}^+$  and  $\text{Cl}^-$  accumulation and photosynthetic rate. Ion concentrations can be detrimental to the integrity of the cell and affect photosynthetic processes directly through membrane damage or enzyme inhibition, if the vacuole can no longer sequester incoming ions. For example, Seemann and Critchley (65) found that high  $\text{Cl}^-$  concentrations (250–300 mM) in the chloroplast of phaseolus correlated with the efficiency of ribulose biphosphate carboxylase oxygenase (RUBISCO). In that study, similar  $\text{Cl}^-$  concentrations were found in both cytoplasm and chloroplasts and vacuole, indicating a breakdown in vacuolar compartmentation.

In contrast to these observations, different types of experiments have found poor correlations between ion accumulation and photosynthetic rates. For example, Tattini and associates (74) observed a full recovery of net photosynthetic rate in olive relieved of a 200-mM NaCl stress, with leaf  $\text{Na}^+$  contents remaining high during relief. Furthermore, Rawson and associates (75) found different relationships between gas exchange and ion concentrations for different leaves and for different salinities.



**Figure 8** Relationship between net CO<sub>2</sub> assimilation rate and ion content (Na<sup>+</sup> and Cl<sup>-</sup>) in leaf 3 of two wheat varieties (Wollaroi and Line 455) grown in 150 mM NaCl. Each data point represents measurements from an individual leaf. (Modified from Ref. 73.)

### E. Molecules Involved in Cell Wall Hardening

The cell elongation of growing leaves is related not only to the turgor, but also to cell wall extensibility and turgor threshold (76). The rate of leaf elongation is regulated or controlled by alterations in any of three parameters: cell wall extensibility, turgor pressure, and yield threshold (76). Thus, the possible causes for the reduction in the longitudinal elongation of leaves under saline conditions may be either decreases in the cell wall extensibility or increases in yield threshold. Under saline conditions, decreases in the cell wall extensibility of maize leaves (32, 77) and increases in the yield threshold of maize leaves (32) may be responsible for the reduction in leaf elongation. However, there is a need for direct



measurements of cell wall extensibility and turgor threshold in the growth zones of grass leaves.

Apoplastic pH is considered to play an important role in cell wall loosening and tissue growth. Several environmental conditions that affect growth were shown to alter apoplast acidification. For example, growth inhibition by water stress is accompanied by an increase in apoplastic pH and a decrease in acidification rate (78, 79). However, Neves-Piestun and Bernstein (80) reported that salinity-induced inhibition of leaf elongation in maize is not mediated by changes in cell wall acidification capacity in growing tissues of leaves.

In 1993 experiments highlighted the biochemical regulation of cell wall extensibility as a key process in controlling growth in plants and led to the identification of a number of proteins that are potentially involved in this process (81). Xyloglucan endotransglycosylase (XET) has been proposed to be involved in the control of cell wall relaxation (81); it catalyses the transglycosylation of xyloglucan, the major hemicellulose polymer that mediates the cross-linking of cellulose microfibrils in the cell wall (82, 83). Significant correlations between high levels of XET activity and tissue elongation have been described [84–86]. Expansins are a family of cell proteins proposed to play a key role in the regulation of tissue elongation, as well as cell wall differentiation (87). However, a study of in vitro wall analysis (88) showed the lack of effect of moderate salinity on apoplastic protein concentrations and enzyme assays in the growth zone of maize leaves in a short-term experiment.

A candidate gene approach was taken in an attempt to identify genes whose expression pattern might function as a marker of tissue elongation and leaf growth of grasses. Reidy and colleagues (89) reported that a detailed analysis of the spatial expression of  $\alpha$ - and  $\beta$ -expansin genes along the leaf elongation zone of *Festuca pratensis* showed no correlation between an expression pattern of these genes and leaf elongation. In another study, however, Reidy and coworkers (90) found that XET-related gene *FpXET1* is a potential marker for leaf elongation in the growth zone of *Festuca pratensis*. However, it is unclear how salinity affects  $\alpha$ - and  $\beta$ -expansin genes in growing tissues.

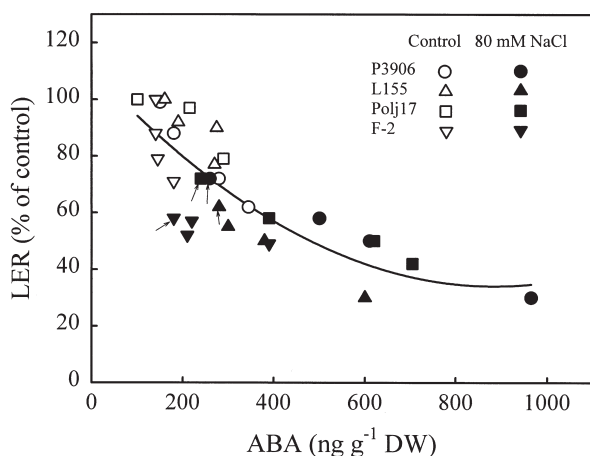
## **F. Cell Elongation and Division Regulated by Signaling and Genes**

Because of the important roles of several hormones in regulating cell elongation, it would not be surprising if stress inhibits cell expansion by changing the concentration of growth-promoting hormones such as abscisic acid (ABA), auxin, cytokinin (CK), gibberellin (GA), and brassinolides (91).

Abscissic acid inhibits leaf expansion (92–95). Several reports have suggested that a signal from the roots communicates with the expanding leaves and growing tissues of the shoots (29), and this may be a similar process to water stress. Experiments within a longer period suggest that ABA levels may regulate cell expansion during salt stress. The increase of ABA concentration in plants by moderate salt stress (96) and water stress (93, 95) is correlated with reduced leaf expansion.

The 2002 study by Cramer and Quarrie (97) showed that ABA concentrations in the leaf growth zone of maize were highly correlated with the inhibition of leaf elongation rate for all four genotypes (Fig. 9). Their results suggest that ABA concentration in the growth zone of leaf is a good predictor of leaf elongation response to salinity.

The characteristics of the leaf growth zone of grasses are similar to those of the root growth zone. The studies by Saab and associates (98, 99) dealt with the effects of ABA content in growing tissues of roots on elongation in the root growth zone at low water potential. After measuring ABA, water content, and elongation of millimeter segments of roots under water stress conditions, Saab and colleagues (98) concluded that a gradient of responsiveness to ABA developed in the cells of the growth zone. The ability of ABA to protect cell expansion of the growth zone, e.g., in the root



**Figure 9** The relation between leaf elongation rate (LER) and the ABA concentration of the growth zone of the third leaf of four genotypes (P3906, L155, Polj 17, and F-2). The ABA concentrations were increased and LER decreased after additions of ABA to control and salt-stressed plants for 23 h. Solid symbols represent the values for 80 mM NaCl-treated plants. Arrows point to values of salt-stressed plants without ABA added. DW, Dry weight. (Modified from Ref. 97.)

at low water potential, decreased with distance from the tip. At low water potential, ABA became more inhibiting to cell expansion with increasing distance from the tip of the root (98). However, it is still not clear how salinity affects ABA content in the leaf growth zone of grasses and how ABA content is directly related to the cell elongation in the growing tissues of grass leaves.

The final size of a leaf is not only determined by the cell size; it is also contributed to by the number of cells. Munns and Termaat (29) reported that cell numbers in grass leaves were significantly reduced by salinity. The zone of cell division in grass leaves is located at the leaf base. For tall fescue, epidermal cell division is restricted to the basal 1.5 to 2 mm, and the division zone of mesophyll cells is extended to 5 to 10 mm above the leaf base (100, 101). Cell division is probably controlled by signaling and candidate genes. However, the connection between stress signaling and control of cell division needs to be better understood (100). A potentially important link between stress and cell division was revealed by induction of a cyclin-dependent protein kinase inhibitor (ICK1) in *Arabidopsis* species by ABA (102). Cell division by reduction of the activities of cyclin-dependent protein kinases that help to drive the cell cycle (91). Salt stress may inhibit cell division by causing the accumulation of ABA, which, in turn, induces ICK1. Furthermore, salinity interferes cell cycle regulatory genes such as *CDC2aAt* and *Arath;CycB1;1* and *Arath; CyA2;1* in *Arabidopsis thaliana* (103).

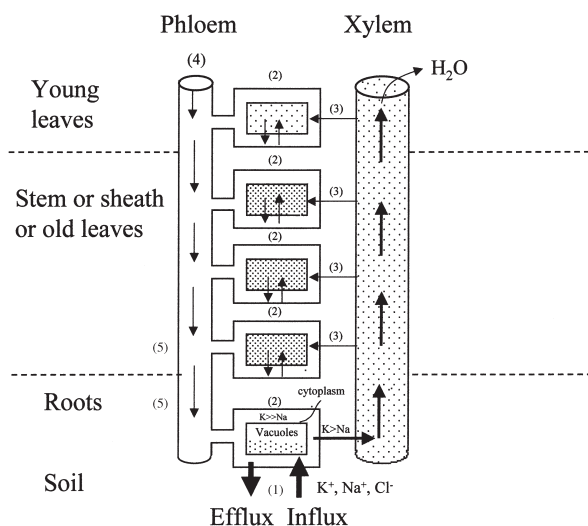
There is evidence that the activity of the protein kinase  $p34^{cdc2}$ , a product of the *cdc2* gene, is involved in the progression of cell cycle in plants. The  $p34^{cdc2}$  kinase activity is necessary to start S and M phases of the cell cycle (104, 105). The  $p34^{cdc2}$  kinase activity and final cell number are decreased in transgenic plants overexpressing a dominant negative mutant of the  $p34^{cdc2}$  kinase (106) and in leaves of wheat plants in water deficit (107). Granier and coworkers (108) showed that the pattern of the spatial distribution of  $p34^{cdc2}$  kinase activity on a per cell basis at the cell division of maize leaves is linked to that of cell division rate. There was a linear relationship between the  $p34^{cdc2}$  kinase activity and cell division rate of a growing maize leaf under water deficit and contrasting temperature conditions.

### III. MECHANISMS OF SALT TOLERANCE: SALT UPTAKE, TRANSPORT, AND COMPARTMENTATION

In principle, salt tolerance can be achieved by salt inclusion or salt exclusion. However, inclusion or exclusion of salt is relative. Excluder plants show a

much lower salt uptake in comparison with includers. In halophytes, high salt tolerance is mainly based on the inclusion of salts and use of salt to lower the osmotic potential for turgor maintenance in aerial plant parts, which facilitates water uptake and transport and lowers the metabolic cost for the production of osmolytes, or  $\text{Na}^+$  in plants can be used for the replacement of  $\text{K}^+$  in various metabolic functions. Therefore, adaptation by salt inclusion requires high tissue tolerance to  $\text{Na}^+$  and  $\text{Cl}^-$  or avoidance of high tissue concentrations. In some halophytes, salt can be excreted through salt glands and bladders.

In glycophytes, which comprise most crop species, exclusion is the predominant strategy; i.e., there is generally an inverse relationship between salt uptake and salt tolerance. Glycophytes restrict the uptake of toxic ions by roots from soils and the movement of toxic ions to the shoot by attempting to control influx into root xylem by root cells and show a selectivity of  $\text{K}^+$  over  $\text{Na}^+$  by roots and a preferential loading of  $\text{K}^+$  rather than of  $\text{Na}^+$  into xylem in order to maintain the high ratio of  $\text{K}^+/\text{Na}^+$  in plants (Fig. 10). Toxic ions can be further transported into the vacuole of cells away from cytoplasm through intracellular compartmentation. Cytoplasmic concentration of  $\text{Na}^+$  is regulated by sequestering  $\text{Na}^+$  from



**Figure 10** Model indicating the key processes of salt transport of salt-tolerant plants: (1), Influx and efflux of salt at root–soil boundary (selectivity,  $\text{K}^+ \gg \text{Na}^+$ ); (2), compartmentation of salt into the vacuole; (3), removal of salt from the xylem; (4), translocation of salt between shoot and root; (5),  $\text{Na}^+$  retained in the upper part of the root system and in the lower part of the shoot (e.g., stem or sheath).

the cytoplasm into the vacuole or across the plasma membrane by sodium and proton exchanger (antiports). The capacity to compartmentalize  $\text{Na}^+$  into the vacuole via antiports is dependent on the activity of  $\text{H}^+$ -ATPase and perhaps the vacuolar  $\text{H}^+$ -pyrophosphatase, which establishes the  $\text{H}^+$  gradient that energizes the transport of  $\text{Na}^+$  against the electrochemical gradient (109). The importance of these various strategies in the overall salt tolerance may vary among plant varieties and severity of salt stress. Tolerant plants can also remove salt from the xylem in the roots to the stem, petiole, or leaf sheaths. In many species,  $\text{Na}^+$  is sequestered in the upper part of the root system and the lower part of the shoot such as stem, leaf sheath, or old leaves, indicating an exchange of  $\text{K}^+$  for  $\text{Na}^+$  by the cells in the stele of the roots or in the vascular bundles in stems and petioles. However, there is little retranslocation of  $\text{Na}^+$  or  $\text{Cl}^-$  in the phloem, particularly in the more tolerant species. This limited retranslocation ensures that salt is not exported to growing tissues of the shoot. Salt-tolerant crop species are able to maintain steep concentration gradients of  $\text{Na}^+$  and  $\text{Cl}^-$  between old and young leaves by restricting the import into the young leaves or apex, for example, in wheat (110) and in maize (111). Excluders adapt to saline conditions by prevention of internal water deficit by enhanced synthesis of organic solutes (e.g., sugars).

Therefore, understanding of the mechanisms of salt tolerance of plants at the molecular level should consider the genes and proteins that may control or regulate salt uptake, compartmentation, translocation, and distribution in plants.

### **A. Genes and Proteins Involved in Salt Uptake and Transport**

There is no specific  $\text{Na}^+$  transporter. Possible mechanisms for  $\text{Na}^+$  entry into roots include permeation of  $\text{Na}^+$  through  $\text{K}^+$  and  $\text{Ca}^{2+}$  transporters, use of  $\text{Na}^+$  transport to energize  $\text{K}^+$  uptake, and  $\text{Na}^+$  selective uptake. Candidate genes for root  $\text{Na}^+$  uptake are found in several  $\text{K}^+$  transporter families: (a) *HKT* transporters, (b) *KUP/HAK/KT* transporters, (c) cyclic-nucleotide-gated channels, and (d) *LCT1*.  $\text{Na}^+$  can be effluxed from the cytoplasm through  $\text{Na}^+/\text{H}^+$  antiporters, driven by the pH gradient across the plasmalemma (112, 113). These transport processes all work together to control the rate of net uptake of  $\text{Na}^+$  by a cell.

The tolerance of most crop species (i.e., glycophytes) requires a high selectivity of  $\text{K}^+$  over  $\text{Na}^+$  at the root–soil boundary and compartmentation of  $\text{Na}^+$  into the vacuole. The accumulation of  $\text{K}^+$  by plant root symplast imposes a substantial energetic cost and requires specialized transport systems (114). The processes of selectivity can be distinguished according to

the activity of proteins (i.e., turnover rate) in the membranes by the three main classes (reviewed by Maathuis and Amtmann [115]):

### 1. Pumps

Transporter fueled by metabolic energy and able to transport substrates against an electrochemical gradient: turnover rates are low, around  $10^2$  per second. A prime example is the ubiquitous  $H^+$ -ATPase. No pumps have been identified in higher plants that directly transport  $K^+$  and  $Na^+$ .

### 2. Carriers

Transport proteins that undergo specific conformational changes during substrate transport: they generally function in transport of substrates against a gradient are energized via coupling to an electrochemical gradient, and have turnovers of  $10^2$ – $10^3$  per second. In plants, “uphill” (high-affinity) accumulation of  $K^+$  is energized through coupling to the “downhill” transmembrane movement of  $H^+$ , proceeding via a  $H^+$ - $K^+$  symporter. Genes as carriers involved in  $K^+$  and  $Na^+$  uptake are presented in Table 2. *KUP-HAK* transporters are extremely selective for  $K^+$  and are competitively blocked by  $Na^+$  when present in millimolar concentrations (Fig. 11) (115, 116). This system clearly creates the potential for severe  $K^+$  depletion when external  $K^+/Na^+$  ratios are low. *HKT1* represents a putative pathway for high-affinity  $K^+$  uptake and low-affinity  $Na^+$  uptake, but its role in uptake of both ions is probably minor compared to that of other systems functioning in plants and may be limited to special cell types (115). In contrast to *HKT1*, *LCT1* may be involved in low affinity  $K^+$  transport.

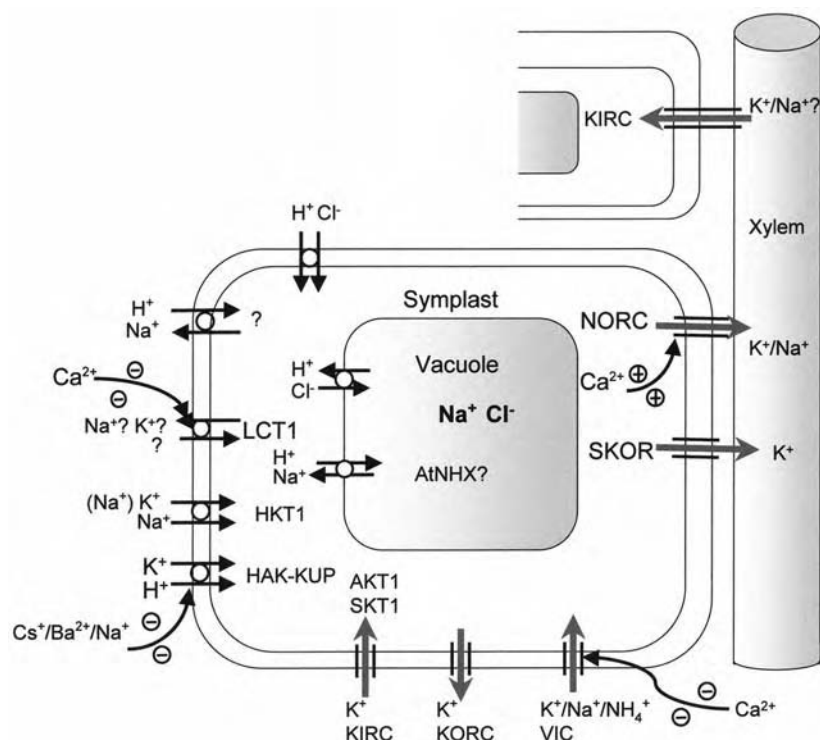
### 3. Ion Channels

Proteins that catalyze the rapid downhill dissipation of transmembrane ionic gradients: turnover rates are  $10^6$ – $10^8$  per second and controlled via opening and closing (gating) of the channel. Channel gating is often under control of the membrane potential. Three types of ion channel, i.e.,  $K^+$  inward rectifying channels (KIRCs),  $K^+$  outward rectifying channels (KORCs), and voltage-independent channels (VICs), have been implicated in the transport of monovalent cations (Fig. 11). They can be distinguished by their ion selectivity and gating behavior. The proportion of time that so-called KIRCs spend in the open state (expressed by their open probability) increases whenever the membrane voltage becomes more negative. And, since channel activation usually occurs at voltage more negative than the equilibrium potential for  $K^+$ , KIRCs allow movement of  $K^+$  only into the cell.  $K^+$  outward rectifying channels (KORCs) have opposite gating

**Table 2** Gene Products Involved in Carrier-Mediated Transport of  $K^+$  and  $Na^+$

Gene product	Species	Expression pattern	Putative localization	Putative function	Coupling
HvHAK	<i>Hordeum vulgare</i>	Root	Plasma membrane	High-affinity uptake of $K^+$	$K^+$ - $H^+$ symport
AtKUP1	<i>Arabidopsis thaliana</i>	Stem, leaves, and flowers	Plasma membrane	High-affinity $K^+$ uptake	$K^+$ - $H^+$ symport
		Root and stem		Dual-affinity $K^+$ uptake	
HKT1	<i>Triticum aestivum</i>	Root cortex	Plasma membrane	High-affinity $K^+$ uptake	$K^+$ - $Na^+$ symport
		Leaves			
		(around vascular tissue)			
LCT1	<i>Triticum aestivum</i>	Root and leaves	Plasma membrane	Low-affinity $Na^+$ uptake	$Na^+$ - $Na^+$ symport
				Low-affinity	
				$K^+$ , $Na^+$ , and $Ca^{2+}$ uptake	Uniport?
AtNHX	<i>Arabidopsis thaliana</i>	Root, stem, leaves, flowers	Tonoplast	$Na^+$ accumulation in vacuole	$Na^+$ - $H^+$ antiport

Source: Adapted from Ref. 115.



**Figure 11** Carrier and channel transport systems that are involved in influx, efflux, translocation, and compartmentation of  $K^+$ ,  $Na^+$ , and  $Cl^-$ . Minus signs, Inhibition, Pws signs, activation. (Modified from Ref. 115.)

characteristics and thus favor  $K^+$  efflux. The open probability of VICs does not change with voltage: all three classes are capable, at least to some extent, of transporting  $K^+$  and  $Na^+$ . Genes involved in  $K^+$  transport are shown in Table 3.

As a result of the negative plasma membrane potential, the electrochemical potential gradient for  $Cl^-$  is uphill into the cell under nonsaline conditions. Uptake can be achieved by means of an active mechanism (117). There is indirect evidence that anion channels do indeed open to allow  $Cl^-$  efflux under salinity. Salt stress-induced enhancement of  $Cl^-$  permeability was noted by Yamaguchita and associates (118). Boursier and Läuchli (119) reported about the extrusion of  $Cl^-$  from the roots of sorghum plants. At very high salinity, the electrochemical potential for  $Cl^-$  has been estimated possibly to be reversed, allowing passive  $Cl^-$  influx into the cells (120, 121).



**Table 3** Genes Encoding Ion Channels Involved in  $K^+$  Transport<sup>a</sup>

Gene	Species	Type	Expression	Inhibitors	Function
<i>AKT1</i>	<i>Arabidopsis thaliana</i>	KIRC	Root cortex	$Cs^+$ /TEA/ $Ba^{2+}$	Low- and high-affinity $K^+$ uptake
<i>AKT2</i>	<i>Arabidopsis thaliana</i>	KIRC	Leaves	?	
<i>KAT1</i>	<i>Arabidopsis thaliana</i>	KIRC	Guard cell	$Cs^+$ / $Ba^{2+}$	Stomatal opening
<i>SKT1</i>	<i>Solanum tuberosum</i>	KIRC	Root Leaf epidermis	$Cs^+$	Root $K^+$ uptake
<i>KST1</i>	<i>Solanum tuberosum</i>	KIRC	Guard cells	$Cs^+$ / $Ba^{2+}$	Stomatal opening
<i>SKOR</i>	<i>Arabidopsis thaliana</i>	KORC	Root pericycle	$Ba^{2+}$ /TEA	Translocation to shoot

<sup>a</sup>KIRC,  $K^+$  inward rectifying channel; KORC,  $K^+$  outward rectifying channel.

Source: Adapted from Ref. 115.

## B. Genes and Proteins Involved in $Na^+$ and $Cl^-$ Compartmentation

Compartmentation of  $Na^+$  in the vacuole prevents building up of high cytoplasmic  $Na^+$  (Fig. 11), which raises the cytoplasmic  $K^+/Na^+$  ratio and contributes to the vacuolar osmotic potential. This is one of the strategies for increasing the salt tolerance of plants. In a number of species a  $Na^+/H^+$  antiport present in the tonoplast allows accumulation of  $Na^+$  in the vacuole by using the transtonoplast  $H^+$  gradient as driving force (115). In *Arabidopsis* sp. the genes for AtNHX1-3 were cloned and all show high homology with yeast and mammalian  $Na^+/H^+$  antiporter (Fig. 11). Expression is observed in all tissues, although functional analysis of the AtNHX products has not yet been carried out and physiological role as well as membrane location are yet to be established. In general, tonoplast  $Na^+/H^+$  antiport activity is induced by growth in NaCl (122). Activity has been reported only for salt-tolerant species such as red beet, sugar beet, barley, and *Plantago* sp. maritime but appears absent in salt-sensitive species such as *Plantago* sp. media. The functioning of  $Na^+/H^+$  antiport is therefore likely to be important in halotolerance, and it is unclear whether glycophytes contain alternative  $Na^+$  accumulating mechanisms or rely solely on passive  $Na^+$  distribution over the tonoplast.

$Na^+$  compartmentation in the vacuole require energy-dependent transport, and an immediate effect of NaCl is vacuolar alkalization [123–125].  $Na^+/H^+$  antiporter activity has been associated with tonoplast vesicles (123, 126), and this association is presumed to be at least partially

responsible for the alkalization. In 1999 plant deoxyribonucleic acids (cDNAs) encoding NHE-like proteins that can functionally complement a yeast mutant deficient for endomembrane  $\text{Na}^+/\text{H}^+$  transporter, NHX1 (127), were isolated. Overexpression of an NHE-like antiporter substantially enhanced salt tolerance of *Arabidopsis* sp., confirming the function of the antiporter in  $\text{Na}^+$  compartmentation.

Tonoplast  $\text{Cl}^-$  transport determinants are predicted to be a channel or a carrier that couples  $\text{Cl}^-$  influx to the  $\text{H}^+$  gradient (Fig. 11). A +50-mV (inside positive) tonoplast membrane potential would be sufficient to facilitate an almost 10-fold concentration  $\text{Cl}^-$  in the vacuole based on electrophoretic flux through an anion-permeable channel (128). Secondary active transport ( $\text{H}^+$  anion antiporter) has also been proposed (129).

Compartmentation of salt in the halophytes depends on regulation of permeability rather than structural proteins. In other words, the differences in the regulatory pathway such as at perception, signaling, or signal transduction rather than structural genes for transport processes with different properties may be the targets for understanding and manipulation in the future (130).

### C. Molecules Associated with Salt Translocation to the Shoot

The glycophytes tend to exclude  $\text{Na}^+$  and  $\text{Cl}^-$  from the growing tissues of shoots by retaining them in the upper part of roots and lower stem, or leaf sheath, or old leaves. This strategy is only successful at low to moderate external salt concentrations and relies on the selective release of  $\text{Na}^+$  into the xylem and its resorption from the xylem stream. Several mechanisms that contribute to the translocation of  $\text{K}^+$  and  $\text{Na}^+$  and some aspects of their regulation have now been identified (reviewed by Maathuis and Amtmann [115]).

The *Arabidopsis* sp. gene *SKOR* (131) encodes a channel protein that is also a member of the Shaker family but displays gating characteristics that favor outward  $\text{K}^+$  flux. The *SKOR* gene has an important role in the translocation of  $\text{K}^+$  to the shoot (Fig. 11). In addition to KORCs, which are highly selective for  $\text{K}^+$  over  $\text{Na}^+$  (including KORCs in maize and barley stellar protoplasts and *SKOR*), a second type of outward-rectifying channel that does not discriminate between monovalent cations (non-selective outward rectifying channels) has been found in patch clamp experiments on barley xylem parenchyma protoplasts (132, 133). Opening of this channel requires micromolar concentrations of cytoplasmic  $\text{Ca}^{2+}$  and creates a potential passage for  $\text{Na}^+$  release into the xylem. At the gene level NORC remains to be identified and its exact role is not yet clear. However, data

suggest a regulatory role for cytoplasmic  $\text{Ca}^{2+}$  in  $\text{Na}^+$  compartmentation between root and shoot, and the process may involve a NORC-mediated  $\text{Na}^+$  release pathway into the stele.

A xylem parenchyma KIRC (132) with relatively low cation selectivity may also be implemented in  $\text{Na}^+$  compartmentation (Fig. 11), since it potentially functions in basal parts of the xylem in  $\text{Na}^+$  resorption.

The salt overly sensitive 1 (SOS1) protein, which is responsible for  $\text{Na}^+$  loading/unloading into xylem, has recently been identified (134). The protein encodes a putative plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter. Loss-of-function mutations in SOS1 confer salt hypersensitivity and SOS1 mutants also cannot grow well under low  $\text{K}^+$  conditions (135). Indirect evidence suggesting that SOS1 may function in  $\text{Na}^+$  unloading is twofold: first, SOS1 is mainly expressed in the pericycle cells surrounding the xylem vessels and also in the veins. This expression pattern in the root is reminiscent of that of SKOC1 (131), which functions in leading  $\text{K}^+$  into the xylem. Second, it was found that when plants were supplied with NaCl,  $\text{Na}^+$  in the xylem sap of SOS1 mutant plants is higher than that in the wild-type plants. This finding suggests that SOS1 may actually prevent  $\text{Na}^+$  from entering the xylem vessel. Interestingly, *SOS1* gene was also expressed in root tips. It is thus likely that SOS1 may have additional functions other than regulating long-distance transport of salts. As the root tip cells are not well developed and are deficient in prototype vacuoles, this expression pattern of SOS1 is consistent with the idea that SOS1 is localized on the plasma membrane, as suggested by its sequence characteristics (133). It is interesting that as the root cells differentiate, the expression of SOS1 becomes restricted to specific cell files, suggesting that positional information is involved in the regulation of SOS1 expression.

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# 6

## Mineral Element Toxicities: Aluminum and Manganese

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### I. ALUMINUM

#### A. Introduction

Aluminum (Al) is a light metal that makes up 7% of the Earth's crust, where it is the third most abundant element after oxygen and silicon. Only at soil pH values below 5.0 (as positively charged ionic species) and above 8.0 (as  $\text{Al}(\text{OH})_4^-$ ) is Al released to the soil solution in concentrations that may be biologically relevant. On acid mineral soils Al toxicity is the most important soil factor determining the composition of the natural vegetation and limiting crop yields. It has been estimated that worldwide on 30–40% of the arable land surface, crop productivity is limited by soil acidity (1). Aluminum may be present in the soil solution of acid soils in monomeric and polymeric forms. Although the potential specific role of the  $\text{Al}_{13}$  hydroxy polymer in Al toxicity is not yet fully understood, the consensus is that monomeric Al species, particularly  $\text{Al}^{3+}$  rather than  $\text{Al}(\text{OH})^{2+}$  and  $\text{Al}(\text{OH})_2^+$ , are the most phytotoxic Al species (2). Organic and inorganic Al complexes are considered not or less phytotoxic (3).

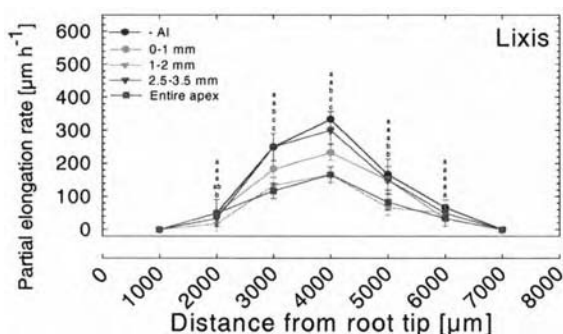
One of the most rapid primary lesions of Al toxicity is an inhibition of root elongation, which can be measured within less than 1 h after exposure of the roots to excess Al. An equally or even more sensitive indicator of Al effects on roots is the induction of callus synthesis, particularly in the root apex (4). Aluminum-induced callus formation is an indicator of sAl sensitivity and a reliable parameter for the classification of genotypes of many plant species for Al resistance. Inhibition of shoot growth by Al

treatment may be regarded as a secondary effect due to Al-induced deficiencies, particularly of Mg, Ca, and P; phytohormone imbalance; and drought stress as a consequence of impaired root growth and root activity.

Although much progress has been made during recent years, the mechanisms of Al-induced inhibition of root elongation and Al resistance are still not well understood. There have been a number of excellent reviews since 1995 summarizing the state of knowledge and addressing knowledge gaps (5–8). Particularly, the relative importance of symplastic versus apoplastic lesions of Al toxicity remains a matter of debate. Rengel (9) and especially Horst (10) focused the attention on the role of the apoplast in Al toxicity in relation to short-term inhibition of root elongation by Al.

## B. Root Spatial Sensitivity

Ryan and associates (11) were the first who unequivocally demonstrated the role of the root apex in the perception of Al toxicity in maize. In a more refined methodological approach Sivaguru and Horst (12) presented evidence that in an Al-sensitive maize cultivar the distal part of the transition zone (DTZ, 1–2 mm) is the most Al-sensitive apical root zone in maize. Application of Al only to the DTZ reduced cell elongation in the elongation zone (EZ) to the same extent as application to the entire 10-mm root apex, whereas application of Al only to the EZ did not inhibit root elongation (Fig. 1; See Ref. 13). These authors also provided evidence that auxin is part of this signal transduction or even the signal mediating the Al effect between DTZ and EZ.



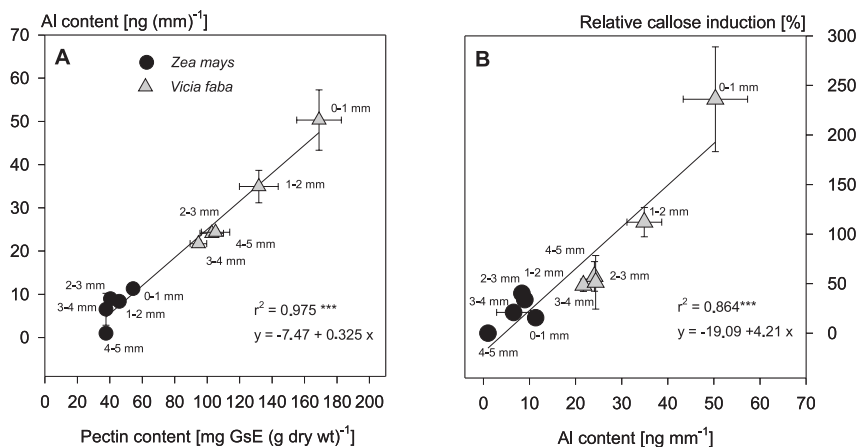
**Figure 1** Effect of Al supply (90  $\mu\text{M}$ , 1 h) to the entire root apex of specific 1-mm root zones on partial elongation rates of 1-mm root segments of the primary roots of the maize cultivar Lixis. Values are means of five independent measurements  $\pm$  standard deviation (SD). Different letters indicate significant differences at  $P < 0.05$  (Tuckey's test). (From Ref 13.)

### C. Aluminum Accumulation and Radial Transport in Roots

Aluminum is accumulated by roots with a rapid initial phase and a lower rate thereafter (14). The primary binding site of  $\text{Al}^{3+}$  is likely the pectic matrix of the cell walls, where negatively charged carboxylic groups have a particularly high affinity for  $\text{Al}^{3+}$ . Short-term Al accumulation by roots is closely related to the pectin content and may explain the differences in Al content between apical root sections of *Zea mays* and *Vicia faba* (Fig. 2).

In fact, the factor responsible for Al binding to pectin is not the pectin content alone but its negative charge determined by its degree of methylation (DM) which is controlled by pectin methyltransferase (PME). Schmohl and colleagues (15) provided evidence that Al accumulation and Al sensitivity of maize cell-suspension cells are modulated by the DM of their cell walls. A modulating role of the DM of root cell walls in Al resistance is supported by the comparison of potato transformants differing in the expression of PME from *Petunia inflata*: transformants with higher PME expression accumulated more Al, produced more callus, and were more inhibited in root growth than the wild type when exposed to Al.

As a result of the strong Al-binding capacity of the cell wall it is not surprising that particularly after shorter periods of Al treatment that induce



**Figure 2** Relationships between (A) pectin and Al content and (B) Al content and relative callose induction (digitonin = 100) of root sections of maize and faba bean. Roots were incubated for 3 h in nutrient solution  $\pm 50 \mu\text{M}$  Al or  $10 \mu\text{M}$  digitonin at pH 4.3. Pectin is given as galacturonic acid equivalents (GsEs). \*\*\*, significant at  $P < 0.001$ .

Al toxicity, most of the Al in the root is located in the cell walls of the outer cortical cells (16). The transport from the rhizodermis to the endodermis was shown to be time-dependent (10 s to 3 h) and no transport of Al into the central cylinder through the fully differentiated endodermis could be observed within 3 h of Al treatment in *Zea mays* and *Vicia faba* (17). An accumulation of Al in the cell wall can also be expected on the basis of the measured low rates of transport of Al through the plasma membrane into the symplast of the model plant *Chara corallina* (18). However, this study as well as the one by Schmohl and coworkers (19) in maize, shows that a rapid transfer of Al from the apoplast to the symplast does occur. Using different techniques Tice (20), Lazof and associates (21), and Vázquez and colleagues (22) demonstrated the accumulation of Al in the symplast in wheat, soybean, and maize, respectively, leading to a rather uniform cellular distribution of Al or even accumulation of Al in the symplast at the expense of the cell wall. The rapid uptake of Al, transfer to the central cylinder, transport to the shoots, and accumulation in the vacuoles of the leaves is a typical feature of Al accumulator plant species such as *Hydrangea macrophylla* (23) and *Fagopyrum esculentum* (24). The reasons for the difference in mobility of Al between Al excluders (most plant species) and includers are not yet understood (25).

#### **D. Apoplastic Solute Flow in Roots**

Aluminum modifies cell-wall composition and properties. Cellulose synthesis was inhibited in favor of callus synthesis in barley (26), and the content of pectin hemicellulose increased in squash root-apices (27), thus enhancing the binding of Al in the cell wall, an effect that was interpreted by the authors as an Al tolerance mechanism. However, as shown previously, binding of Al to pectin appears to be more closely related to Al sensitivity in maize. Cross-linking of pectins by  $\text{Al}^{3+}$ , thus reducing cell-wall extensibility, has been claimed to be responsible for the inhibition of root elongation by Al (28), but direct application of Al to the elongation zone was not inhibitory to root elongation in maize (see Fig. 1). Therefore, in maize a direct interference of Al with the process of cell elongation as primary target of  $\text{Al}^{3+}$  appears less probable.

#### **E. Impairment of Membrane Functions and Related Physiological Disorders**

Aluminum rapidly affects not only cell-wall but also plasma-membrane properties. Interaction of Al with membrane lipids and proteins induces

modifications of its structural properties such as fluidity (29). Such structural changes in membrane properties are among the prerequisites, in addition to an increase in the cytosolic  $\text{Ca}^{2+}$  activity, for the induction of callus synthesis (30), a most sensitive response of root apices to Al (see previous discussion). Binding of Al to the plasma membrane alters its electrical properties. In *Curcubita pepo* Ahn and coworkers (31) found a reduction of the plasma-membrane surface negativity. Also, in most studies Al supply rapidly induced membrane depolarization, specifically in the most Al-sensitive root zone (DTZ) (32). This may be related to an inhibition of the  $\text{H}^+$ -adenosine triphosphatase ( $\text{H}^+$ -APTase) activity that may lead to a disturbance of the  $\text{H}^+$  homeostasis in the cytosol (33). As a result of the changes in plasma-membrane properties by Al, its ion-transport properties are affected. In *Glycine max*, Al treatment led to a rapid decrease of  $\text{K}^+$  efflux without changing  $\text{K}^+$  influx (34). In *Triticum aestivum* the Al-enhanced release of malate was charge-balanced by a release of  $\text{K}^+$  (35). Al-induced impairment of membrane functions may be related to Al-enhanced oxidative stress through the formation of reactive oxygen species (ROS) leading to lipid peroxidation (36) and protein oxidation (37) mediated by  $\text{Ca}^{2+}$  signaling (see later discussions). Among the identified genes that are expressed after Al treatment are several oxidative-stress related genes. Transformation of *Arabidopsis thaliana* with such genes conferred Al resistance (38). However, oxidative stress in roots appears not to be the primary cause of Al-induced inhibition of root elongation, because in most cases it could be observed only after prolonged Al treatment (37,39). But sustained Al resistance may require protection mechanisms against oxidative stress.

In spite of these changes in plasma-membrane structure and functions, the finding is no indication that at physiological Al concentrations, a severe disruption of plasma-membrane functions is a prerequisite for inhibition of root elongation and callus formation must be stressed (40). It appears that Al triggers signal transduction pathways, leading to the observed symplastic physiological disorders. In this regard the effect of Al on cytosolic  $\text{Ca}^{2+}$  seems to play a crucial role (41). An increase in cytosolic  $\text{Ca}^{2+}$  as immediate response to Al treatment has been demonstrated in different plants. The source of  $\text{Ca}^{2+}$  is likely the apoplastic  $\text{Ca}^{2+}$  pool, because  $\text{Ca}^{2+}$  bound in the apoplast is liberated by  $\text{Al}^{3+}$  and the change of the plasma-membrane potential results in an activation of  $\text{Ca}^{2+}$  channels. However, the triggering of a release of  $\text{Ca}^{2+}$  from symplastic  $\text{Ca}^{2+}$  pools by Al cannot be excluded. Increasing cytosolic  $\text{Ca}^{2+}$  can explain three cellular distortions: callus formation, disorganization of the cytoskeleton, and formation of ROS. An increase in cytosolic  $\text{Ca}^{2+}$  is one of the prerequisites for the induction of callus synthesis by different elicitors. Aluminum-induced



alterations of the cytoskeleton have been reported (32). Although a direct effect of cytosolic Al on the cytoskeleton cannot be ruled out, an interaction of apoplastic Al with the cell wall plasma membrane–cytoskeleton continuum appears more likely (42).

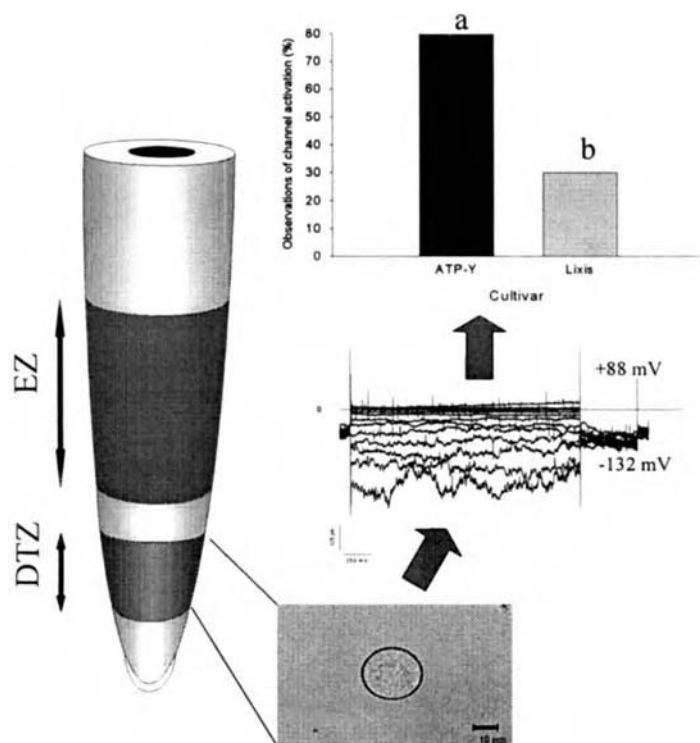
## F. Aluminum Resistance

As shown previously Al readily attaches to binding sites in the apoplast and the plasma membrane in the most Al-sensitive sites of the root apex. Since this attachment may lead to enhanced transport of Al to the symplast and/or to impairment of root growth and functions (see earlier discussion), one not only can expect but has to postulate that reduced binding of Al in the apoplast is a prerequisite for Al resistance. Kinraide and associates (43) were able to explain inhibition of root elongation by  $\text{Al}^{3+}$  in the presence of competing cations, particularly  $\text{Ca}^{2+}$  but also  $\text{H}^{+}$ , on the basis of the computed cation distribution on a negatively charged root membrane surface. Grauer and Horst (44) reached comparable conclusions based on similar but conceptually different approaches. A lower root cation-exchange capacity (CEC) as a measure of cell-wall negativity has been reported in plant species adapted to acid soils with high Al supply. However, across a large range of plant species a clear relationship between root CEC and Al resistance does not exist.

In addition to the cell wall, the plasma membrane contributes to the negativity of the apoplast. Wagatsuma and colleagues (45) related differences in Al resistance between plant species to the plasma membrane negativity of protoplasts, and Yermiyahu and coworkers (46) ascribed the higher Al sensitivity of a *Triticum aestivum* cultivar to its higher plasma-membrane negativity compared to that of the Al-resistant cultivar.

An even more effective way to reduce the impact of Al on apoplastic functions is the release of Al complexing solutes, particularly organic acid anions from the Al-sensitive apical root zone (47). Using the patch-clamp technique, it is now well established that the Al-induced release of malate in *Triticum aestivum* (48) and citrate in *Zea mays* (49) is mediated by plasma-membrane anion channels. In Al-resistant cultivars the frequency and magnitude of the Al-induced anion currents were larger (Fig. 3; see Ref. 48).

The role of the metabolism of organic acids in Al resistance is still a matter of discussion. In most studies a clear relationship between the root content and release of organic acids did not exist (47). Also, the activities of enzymes involved in the synthesis of organic acids did not differ significantly between Al-resistant and Al-sensitive genotypes. However, on the basis of a detailed study of release from, and content of, specific 1-mm apical root sections, Kollmeier and Horst (50) showed that an Al-sensitive maize



**Figure 3** Application of the patch-clamp technique to protoplasts isolated from the apical root cortex. Preincubation of intact roots with  $90\text{ }\mu\text{M}$  Al for 1 h induced a citrate- and malate-permeable large conductance anion channel in protoplast isolated from the distal transition zone (DTZ) but not of the elongation zone (EZ). Anion channel activity was induced in 80% of the DTZ protoplasts from the Al-resistant maize cultivar ATP-Y but in only 30% of the Al-sensitive cultivar Lixis. Different letters (a, b) indicate significant differences at  $P < 0.05$  (Fisher's exact test). (Adapted from Ref. 50.)

cultivar was not capable of maintaining the level of citrate in the apical root sections in spite of a lower citrate release rate. This finding was in agreement with a general trend of Al-enhanced activities of enzymes involved in citrate synthesis such as malate dehydrogenase (MDH) and phosphoenolpyruvate decarboxylase (PEPC) in the Al-resistant cultivar and of citrate degrading aconitase in the Al-sensitive cultivar. The strongest evidence for an involvement of organic acid synthesis in Al resistance is from studies using transgenic plants with modified organic acid metabolism. De la Fuente and associates (51) reported on transgenic tobacco with largely

enhanced citrate accumulation and exudation through expression of a bacterial citrate synthase in the cytoplasm that conferred Al resistance to these plants. However, attempts by other authors to enhance citrate synthesis and to improve Al resistance of tobacco using the same gene were not successful (52). Although the overexpression of not only citrate synthase but also MDH and PEPC was reported to enhance Al resistance of plants, it appears that the key to Al-enhanced release of organic acid anions and thus Al resistance are the expression, activity, and control of plasma-membrane anion channels rather than the synthesis of organic acids.

There are indications that in addition to organic acid anions the release of polypeptides and phenols may be involved in genotypic Al resistance in wheat and maize, respectively.

However, complexation of Al through root exudates and thus reduced Al accumulation in the root apices can explain Al resistance, for instance, in the tropical pasture grass *Brachiaria decumbens*, though not in all plant species (53). Among the most Al-resistant plant species are Al includers, such as *Camellia sinensis* and *Hydrangea macrophylla*. In these plants Al resistance is attributed to the detoxification of Al by organic acids (54), particularly in the vacuoles, where Al may precipitate in association with silicate and/or phosphate (22). There is also increasing evidence that phenols may be involved in the sequestration of Al (55).

## II. MANGANESE

### A. Introduction

Worldwide, manganese (Mn) excess represents an important factor limiting growth and crop yields on acid, insufficiently drained soils with low redox potential (56,57): the effect has been also observed under conditions such as drought, heat, and after steam sterilization of soils. Soil amelioration such as liming and soil drainage often are not economic and only partly successful (58). Several developmental, environmental, and nutritional factors influence Mn toxicity in plants, e.g., leaf age, temperature, light intensity, silicon (Si) supply, and source of nitrogen (59). Furthermore, great differences in Mn tolerance exist between plant species and cultivars within species. A particular high genetic variability was observed in the Mn-sensitive legumes cowpea (*Vigna unguiculata*) and soybean (*Glycine max*). First visible Mn toxicity symptoms in these species are brown spots on older leaves, followed by chlorosis, necrosis, and leaf shedding. The brown spots represent local accumulations of oxidized Mn ( $\text{Mn}^{\text{IV}}$ ) and oxidized phenols in the cell wall, especially of the epidermis (60).

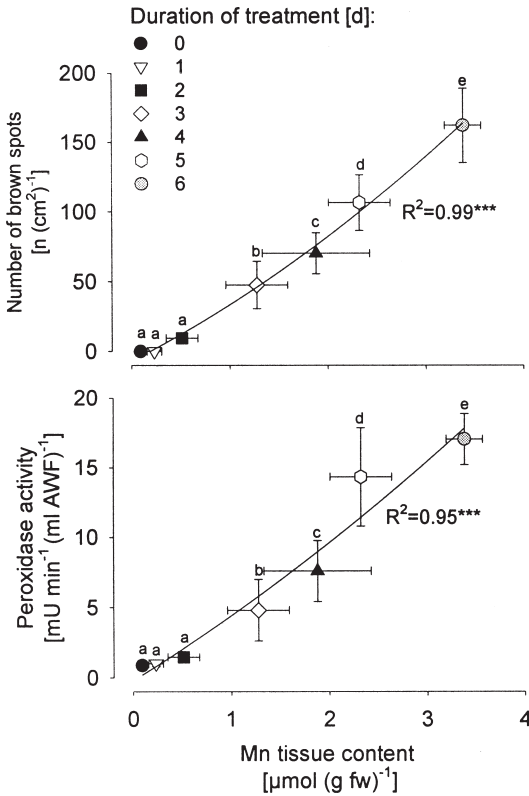
The physiological–molecular mechanisms of Mn toxicity and resistance are still unknown. To create a better understanding of Mn toxicity in plants, changes in the leaf apoplast in response to Mn stress and their specific role on Mn toxicity and relevance for Mn tolerance are specifically discussed.

## **B. Manganese Toxicity—Manganese-Induced Changes in the Apoplast Proteome**

The oxidation of  $Mn^{II}$  in the apoplast has been proposed as a key reaction leading to Mn toxicity (59), because  $Mn^{III}$  may react as a powerful oxidant of proteins and lipids. Kenten and Mann (61) found a close relationship between the oxidation of Mn in the presence of peroxidase (POD) and phenols, and the activation of PODs by excess Mn was documented by Horst (59) and Horiguchi and Fukumoto (62). Peroxidase is often used as a physiological marker for plant-stress responses with an apparent lack in specificity. But the stimulating effect of Mn on  $H_2O_2$ -producing PODs (63, 64) particularly indicates a specific role of Mn in the function of PODs in the apoplast. A more detailed analysis of PODs of several fractions of the leaf tissue was used to verify the specificity of the response of PODs to Mn excess (65). The PODs extracted from several fractions of the leaf tissue were significantly activated by Mn treatment in the Mn-sensitive cowpea cultivar TVu 91, whereas the Mn-tolerant cv. TVu 1987 showed no change in POD activities due to excess Mn supply. The soluble peroxidases of the leaf apoplast, extracted by collecting apoplastic washing fluid (AWF) by using the vacuum-infiltration technique, were most affected by Mn treatment compared to PODs from the cytoplasm and bound to the cell wall (65). This finding is in agreement with results showing that extracellular PODs respond more sensitively to oxidative stress induced by, e.g., ozone exposure, than cytosolic PODs (66). Furthermore, activity of PODs in the AWF increased significantly and simultaneously with the formation of characteristic brown spots on leaves (Fig. 4; see Refs. 65, 67).

These findings suggest a close relationship between freely mobile apoplastic PODs and the oxidation of Mn and phenols in the apoplast. Electrophoretic separation of proteins in the AWF by Blue-native polyacryl gel electrophoresis (PAGE) demonstrated a strong release of PODs into the apoplast with increasing duration of Mn treatment (67). Released PODs were identified by nano liquid chromatography mass spectrometry (LC-MS/MS) as acidic, anionic PODs with a molecular mass around 32 kd.

Peroxidases were also proposed to produce  $H_2O_2$  in the apoplast necessary for lignification (63,64). The  $H_2O_2$  production and consumption are probably catalyzed independently by different PODs, as suggested by



**Figure 4** Relationships among Mn tissue content, the density of brown spots, and the activity of freely mobile peroxidases from the leaf apoplast of cowpeas. Plants of cowpea cultivar TVu 91 (Mn-sensitive) were precultured hydroponically in a growth chamber for at least 13 days. Mn supply (50 μM) was increased for up to 6 days, whereas control plants received 0.2 μM continuously. AWF, apoplastic washing fluid. (From Ref. 67.)

Mäder and colleagues (68). Apoplastic PODs in the AWF of cowpea were able to oxidize reduced Nicotinamide adenine dinucleotide (NADH) accompanied by the formation of H<sub>2</sub>O<sub>2</sub> (69). Both were increased by Mn treatment. The oxidation of NADH was enhanced in the presence of Mn and *p*-coumaric acid. The drastic increase of Mn concentration in the apoplast may cause a direct activation of NADH PODs with subsequent formation of H<sub>2</sub>O<sub>2</sub>. Since a Mn-induced H<sub>2</sub>O<sub>2</sub>-production was observed in washed intact leaf segments (70) and in cell-culture medium of cell suspensions of soybean (Fecht-Christoffers and colleagues, unpublished),

formation of  $\text{H}_2\text{O}_2$  by PODs stimulated by phenols and Mn may be the initiation of Mn toxicity with subsequent reduction of  $\text{H}_2\text{O}_2$  by PODs, accompanied by an oxidation of phenols and probably  $\text{Mn}^{\text{II}}$ .

The release of PODs in the apoplast was accompanied by the secretion of a range of further proteins into the apoplast (67). The Mn-induced proteins showed high sequence homologies to wound-induced proteins and pathogenesis-related (PR) proteins.

The PR-like proteins are induced not only by biotic stresses but also by a wide range of environmental factors (71). The expression of these proteins could often be related to the presence of plant signalling molecules (72). Different forms of stress applied to plants did not always result in similar transcriptional changes, indicating the presence of multiple pathways of gene regulation in response to abiotic stresses. Because of the complicated crosstalk in the signaling pathways within plants, the identification of primary effects of stresses is difficult. Since enhanced senescence, ethylene production, and indole acetic acid (IAA) oxidation are typical features of advanced stages of Mn toxicity (73,59), it appears that the induction of PR-like proteins and particularly the Mn-induced release of PR-like proteins into the leaf apoplast are secondary general stress responses of plants.

### C. Manganese Tolerance—The Role of Organic Acids

Genotypic Mn resistance in most plant species is primarily due to a higher Mn tolerance of the shoot tissue (59). Genotypic differences in Mn tolerance can be easily assessed by using the appearance of Mn toxicity symptoms, which are brown speckles on older leaves (74). The induction of callus formation by Mn proved to be an even more sensitive parameter for Mn sensitivity (75). In Mn-tolerant leaf tissue local accumulation of Mn is prevented by a more homogeneous distribution (59). The reason for this more homogeneous distribution is still unknown. Results of mark by Wissemeier and Horst (76) indicate that compartmentation of Mn is of great significance in Mn tolerance of cowpea genotypes. Accumulation of Mn in the vacuoles may lead to a more homogeneous distribution of Mn on tissue and cellular levels. At the same time Mn sequestration preferentially by organic acids such as citrate and oxalate, forming strong complexes with heavy metals, functions in detoxification. Such a detoxification mechanism has been proposed for Zn and Cd (77–79) and Mn in a Mn hyperaccumulator (80). Since the Mn toxicity symptoms indicate a special role of Mn oxidation in the cell wall, sequestration of Mn in the apoplast may also be important for Mn leaf-tissue tolerance. However, detailed studies on the relationships between Mn accumulation in the leaf vacuoles and that in

the apoplastic washing fluid in two cowpea cultivars greatly differing in Mn tolerance did not suggest that accumulation of Mn in the vacuoles and/or apoplast and complexation through organic acids play a fundamental role in Mn leaf-tissue tolerance in cowpea (81). Also, the considerably enhanced Mn tolerance of cowpea plants supplied with Si could not be explained by sequestration of Mn by organic acids in the vacuoles or the apoplast (81). However, molecular biological studies in 2000 and 2003, respectively show that in tobacco (82) and yeast (83) the expression of the vacuolar transporter AtCaX2 confers enhanced Mn tolerance, indicating that in these organisms the accumulation and sequestration of Mn in the vacuoles are important components of Mn tolerance.

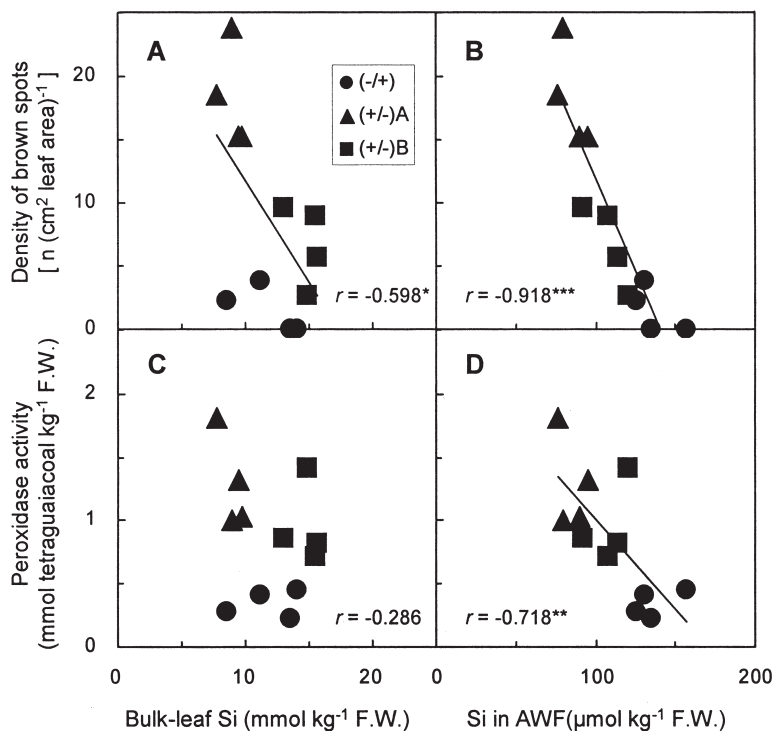
#### **D. Manganese Tolerance—the Role of Silicon**

It is well established that silicon (Si) greatly improves the Mn tolerance of many plant species including rice, barley, bean, and cowpea (81). The physiological–molecular background of this effect is not yet well understood.

The relationship between the manganese (Mn) and silicon (Si) concentrations in the apoplastic washing fluid (AWF) and the severity of Mn toxicity symptoms were investigated in the leaves of the Mn-sensitive cowpea cultivar TVu 91 in solution-culture experiments (84,85). The expression of Mn toxicity symptoms was prevented when 1.44 mM Si (applied as potassium silicate) was supplied together with 50  $\mu$ M Mn (supplied as  $\text{MnSO}_4$ ). However, distinct Mn toxicity symptoms were observed in plants pretreated with 1.44 mM Si and then exposed to 50  $\mu$ M Mn without concurrent Si supply. In both Si treatments, plants had lower Mn concentrations in the AWF and higher amounts of adsorbed Mn on the cell walls than the plants treated at 50  $\mu$ M Mn without Si supply. Inactivation of Mn in the cell walls by Si has been regarded as the main mechanism of Si-induced alleviation of Mn toxicity in cucumber (86). However, in cowpea the severity of Mn toxicity symptoms and the Mn-enhanced guaiacoal POD activity in the AWF of these plants were not significantly correlated with the Mn and Si concentrations in AWF but were highly significantly correlated with the Si concentrations in AWF (Fig. 5; see Ref. 85). These results suggested that Si supply alleviated Mn toxicity symptoms not only by the decrease of apoplastic Mn concentration and the increased adsorption of Mn on the cell walls but also by the soluble Si in the apoplast.

#### **E. Manganese Tolerance—Phenolic Compounds**

The biosynthesis of phenolics is activated by a wide range of environmental, hormonal, and nutritional factors, e.g., light, stress, growth regulators, and

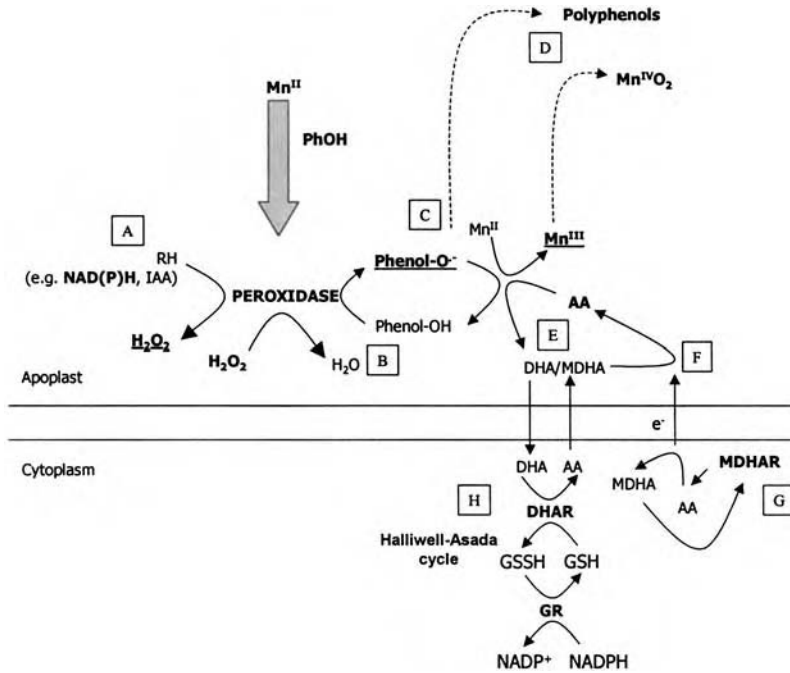


**Figure 5** Linear relationships between Si concentration in the bulk leaf or in the apoplastic washing fluid (AWF) and densities of brown spots (A, B) or peroxidase activities in the AWF (C, D). \*, \*\*, \*\*\*, significant correlations at the 5.0%, 1.0%, and 0.1% levels, respectively. FW, fresh weight. (From Ref. 85.)

the levels of nitrogen, phosphorus, and boron, and several functions have been attributed to phenolic compounds in plant tissues (87). Among these, phenolic compounds were proposed to enhance metal tolerance by chelating metal ions (88). Furthermore, phenolic compounds have a significant effect on the functionality of PODs (63,64,89).

The stimulating effect of Mn on phenol content in the tissue (90) supports the postulated release of phenolic compounds into the apoplast at excess Mn (91). In cowpea, concentrations of apoplastic phenols increased as a result of Mn treatment and were positively correlated with Mn tissue content. A release of phenolic compounds into the extracellular space was also associated with the release of PODs, assuming a POD-catalyzed phenol oxidation as a response to several stresses (92). A significant Mn-enhanced release of phenolics was only observed in a Mn-sensitive cowpea cultivar





**Figure 6** Proposed reactions in the leaf apoplast of the Mn-sensitive cowpea cultivar TVu 91 caused by excess Mn. A, Peroxidases are directly stimulated by MnII and available apoplastic phenols with subsequent formation of H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> in the apoplast serves as a signal inducing a cascade of mechanisms in the apoplast and cytoplasm, leading to callus formation and the release of proteins, organic acids, and phenols in the apoplast. B, Aliquots of H<sub>2</sub>O<sub>2</sub> are reduced by peroxidase with subsequent oxidation of phenolic compounds. C, Intermediates of phenol oxidation (phenoxyl radicals) are oxidizing MnII, causing the formation of MnIII. D, MnIII disproportionates to MnII and MnIV; accumulation of MnIV and oxidized phenols in the cell wall causes the formation of brown spots. E, Ascorbate in the apoplast is involved in peroxidase- (POD)-catalyzed redox reactions and is oxidized to monodehydroascorbate (MDHA) and dehydroascorbate (DHA). F, For regeneration of MDHA in the apoplast, abscisic acid (AA) is oxidized to MDHA in the cytoplasm and reduction equivalents are transported into the apoplast. G, Regeneration of cytoplasmic MDHA was caused by MDHA reductase (MDHAR). H, DHA is regenerated via the Halliwell–Asada cycle by the enzymes DHA reductase (DHAR) and glutathione reductase (GR) in the cytoplasm. GSSH, reduced glutathione; AA, abscisic acid; NADP<sup>+</sup>, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; GSH, reduced glutathione; IAA, indoleacetic acid; NAD(P)H, reduced nicotinamide adenine dinucleotide phosphate; RH.

(Fecht-Christoffers, unpublished), indicating that the release of phenols reflects a response to excess Mn in Mn-sensitive leaf tissues rather than a mechanism of Mn tolerance. It appeared that the phenol content and particularly the phenol composition of the apoplastic fluid are important for Mn toxicity and tolerance with regard to chelation and thus detoxification of Mn and/or as cofactors of POD-catalyzed reactions.

In conclusion, on the evidence presented, Fig. 6 schematically shows the proposed linkage among peroxidase-mediated  $H_2O_2$  production, Mn and phenol oxidation, and the role of ascorbic acid and phenols involved in modulating Mn tolerance.

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# 7

## Herbicides

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### I. INTRODUCTION

Since the first organic herbicides were developed for weed control in the late 1940s and early 1950s, we have seen the emergence of a vast arsenal of ever more specific and more active organic herbicides. Starting with the auxinic phenoxy acids and then sequentially adding photosynthesis-inhibiting phenylurea and *s*-triazine compounds, the bleacher amitrole, and an ever growing number of new structures, about 400–500 organic herbicide compounds are known to have been commercialized. These are divided into ca. 70 chemical structure groups. Of these, however, only 272 are presently registered (1). In this chapter, 148 herbicidal compounds acting at 23 different primary (molecular) targets are presented, including some new targets that have not yet led to commercial products (Table 1). This selection may be compared to the classification of herbicides according to their modes of action and to the structures that the Herbicide Resistance Action Committee (HRAC) has presented at its Internet site (2). Common and chemical names and further details on the chemical and physical properties of herbicides and pesticides in general may be found in “The Pesticide Manual” (3). A compilation of the scientific and common names of all main weed and crop plants known worldwide together with approved five-letter computer codes is available (4). The herbicide modes of action have been summarized in several sources (5–11).

The 23 primary targets presented in this article are grouped according to their subcellular location in Table 1. In the frequent cases of target

**Table 1** Herbicide Targets Arranged According to Their Cellular Locations

Target location and name	Abbreviation	Function in metabolism
<b>Chloroplasts</b>		
Protoporphyrinogen oxidase	PPO	Chlorophyll synthesis
D1 protein in photosystem II	D1	Photosynthetic electron transport
Photosystem I	PS I	Ferredoxin reduction
Deoxyxylulose-5-phosphate reductoisomerase	DXPRI	Isoprenoid synthesis
Farnesylpyrophosphate synthase	FPPS	Isoprenoid synthesis
4-OH-phenylpyruvate dioxygenase	HPPD	Synthesis of $\alpha$ -tocopherol
Phytoene desaturase	PDS	Synthesis of carotenoids
$\zeta$ -Carotene desaturase	ZDS	Synthesis of carotenoids
Lycopene cyclase	LCC	Synthesis of carotenoids
Glutamine synthetase	GS	Supply of amino groups
Acetolactate synthase	ALS	Synthesis of branched chain amino acids
Acetohydroxy acid reductoisomerase	AHRI	Synthesis of branched chain amino acids
Enolpyruvylshikimate-3-phosphate-synthase	EPSPS	Synthesis of aromatic amino acids
Acetyl-CoA carboxylase	ACCase	Synthesis of fatty acids
<b>Mitochondria</b>		
Transmembrane proton gradient		Respiratory adenosine triphosphate synthesis (ATP)
<b>Cytoplasm</b>		
Nitrate reductase	NR	Supply of reduced nitrogen
Asparagine synthetase	AS	Supply of nitrogen transport form
Tubulin	TUB	Polymerization of microtubules
Microtubule organizing center	MTOC	Cell division and wall growth
Very-long-chain fatty acid elongase	VLCFAE	Membrane morphological structure, waxes
Dihydropteroate synthase	DHPS	Synthesis of folic acid
<b>Plasma membrane</b>		
Auxin binding protein	ABP	Regulation of cell extensibility
Cellulose synthase	CS	Synthesis of cellulose microfibrils

isoenzymes and/or location at several subcellular sites, the herbicidally most important site is chosen. The same argument applies to the entries for the function in metabolism. It is evident that the chloroplasts are the most important organelles for herbicide action. This importance reflects the central function of chloroplasts in plant metabolism, which is related to the immediate availability of energy and reduced carbon and nitrogen compounds in this organelle in light. This situation also explains why most herbicides require the presence of light for their herbicidal action.

For practical use in the field, preemergence and postemergence herbicides are differentiated. Postemergence herbicides can be adapted to the needs of late weed situations in the field and can therefore be applied on demand. Preemergence application is used more often because more flexibility is available to select an application time, e.g., either in fall or in early spring. Postemergence application means leaf and root uptake, and the demands on crop selectivity are quite high. After preemergence application, root uptake is most important. Some herbicide may be taken up by the emerging shoot when growing through the soil. The herbicides are generally translocated in the xylem from the roots to the leaf, but postemergence herbicides should also be phloem-mobile in order to control new growth as well. Contrasting with selective herbicides developed for specific crops, nonselective herbicides are used on places such as rights of way and in orchards or vineyards. In the latter, placement selectivity is realized where the shallow rooting weeds are controlled, whereas the deeper root horizons of the woody plants are not reached by the herbicide. Of course, the herbicide must not leach into deep soil for this type of application.

In some fields where the same herbicide has been repeatedly used over several years, resistant weed populations have developed. An important prerequisite for herbicide resistance is chemical stability in the environment, providing herbicidal action of the selecting compound for several months or longer. The first resistance cases appeared with *s*-triazine herbicides in the 1970s. Meanwhile, herbicide-resistant weeds exist for most of the larger herbicide groups. The mechanism of resistance is often a point mutation leading to the loss of herbicide binding at the target site, but increased herbicide metabolism rates also occur, often by increased activities of cytochrome P450 monooxygenase enzymes. Examples of the first mechanism may be found in this text for the photosystem II (PS II) and the acetolactate synthase inhibitors. A third mode of resistance is an increased expression of the target enzyme. A 10-fold increase in the amount of target protein means a doubling of the required herbicide rate. This mode of resistance was used in some of the early genetically modified glyphosate-resistant crops, but they were not commercialized. A current list of all weed resistance cases so far documented worldwide is available on the

Internet (12). Presently these are a total of 151 species. Examples for some targets are 65 species for acetolactate synthase (ALS), 63/20 (*s*-triaziones/ureas) for the D1 protein, 25 for acetyl-coenzyme A carboxylase (ACCase), 25 for PS I, and 10 for tubulin (dinitroanilines). Literature and more information on herbicide resistance may be found at the HRAC Internet site (2).

The search for new herbicides up to now was more or less by a random approach. The random synthesis of similar and new chemical structures and their testing on selected weed and crop plants by a greenhouse screening procedure were indeed very successful. But only after the molecular target became known from subsequent research and could also be tested *in vitro* were guided synthesis and target-oriented optimization possible. One successful early example of this approach was the testing of photosystem II inhibitors in isolated chloroplast thylakoid membranes, generating  $pI_{50}$  ( $-\log_{10}$  of the molar concentration for 50% inhibition) values. These data, together with physicochemical parameters such as the log *P* value for lipophilicity, helped in understanding molecular properties for good herbicidal action (13, 14). A prerequisite for this approach also was a choice of many herbicidally active structures in the series. This was the case for photosystem II and acetolactate synthase inhibitors, for which hundreds of good herbicides have been found. Only in these cases of many similar good herbicides has it been possible to find examples with superior selectivity in a major crop. Selectivity in crop plants is in most cases mediated by herbicide-detoxifying enzymes. Prominent detoxification mechanisms are glutathione conjugation, e.g., in corn (15), and cytochrome P450 monooxygenation followed by conjugation with sugars, e.g., in cereals (16).

In other cases only one single structure is herbicidally active. Chemical derivatization or synthesis of even closely similar structures leads to a total loss of herbicide action. Examples for the latter situation are glyphosate and glufosinate, which consequently could originally only be developed as non-selective herbicides. Recently, however, these two herbicides have been used for the development of herbicide-resistant crops. A few remarks relating to the use of the terms *tolerance* and *resistance* may be helpful. The term *resistance* generally means a high level of herbicide selectivity: i.e., several times the amount of herbicide required for the herbicidal action is needed before damage to the crop plant occurs (i.e., a 5–10 × safety factor). *Tolerance* means a 2–3 × safety factor, a frequent situation in the development of new herbicides selected from greenhouse screening. However, a resistance-type safety factor may also occur for herbicides found by the classical random approach, e.g., in the case of the ACCase inhibitors. The more adequate term here would be *insensitive crop*. The resistance mutations described fit this description in that they frequently show a greater than 10 × safety factor, e.g., in the *s*-triazine-resistant weeds.

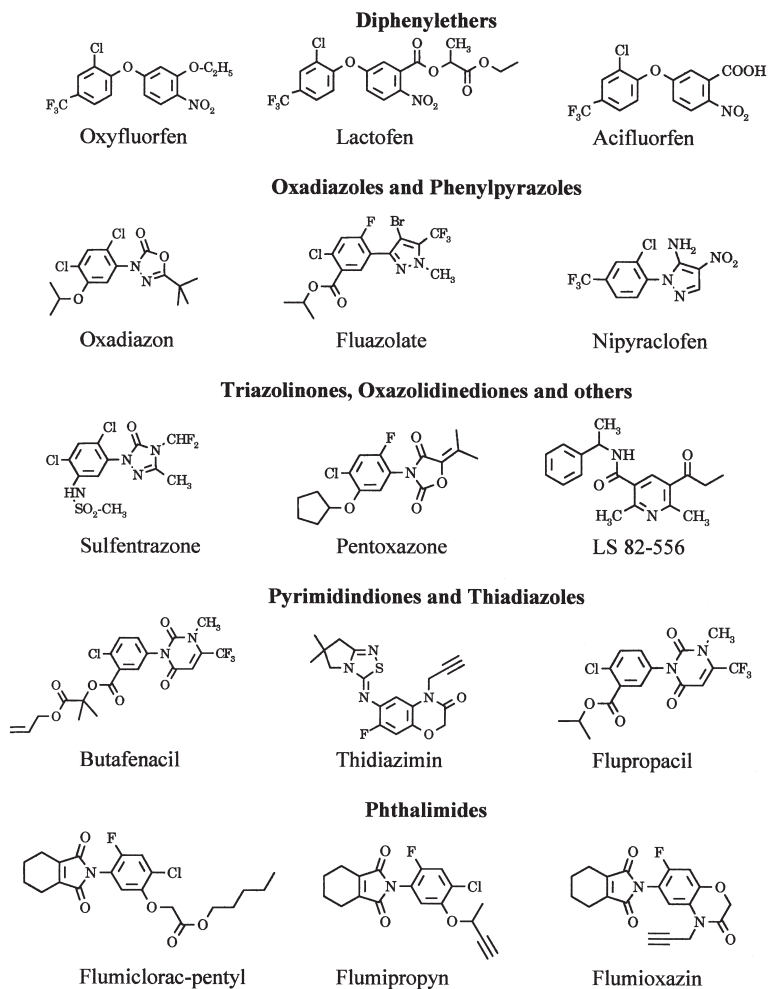
The term *selectivity* is used for the description of the classical situation in crop plants, meaning a sufficient safety factor between crop and weed sensitivity for an application as a herbicide. According to an HRAC definition, *resistance* specifically means the situation in the field when new and stable/heritable weed populations that are no longer controlled by the herbicide used previously have evolved. The use of the terms *selectivity*, *tolerance*, and *resistance* has varied considerably.

Presently, we see the emergence of a new approach to finding new herbicide targets by exploitation of the information supplied by molecular genomics (1). The starting point is made by the newly available sequence of the complete genome of the cruciferous weed *Arabidopsis thaliana*. Of the ca. 25,000 genes expected to constitute this genome, a large fraction will be exploited by knock out mutants for their possible use as herbicide targets. From these, the most promising (probably between several hundred and 2000) must be subjected to a functional analysis in order to find out their function in plant metabolism. Eventually, some 100 gene products/enzymes are expected to be subjected to testing in high-throughput screening (HTS) systems. The HTS methods employ robot technology to test  $10^5$  to  $10^6$  compounds for inhibitory potency, eventually leading to new herbicides with new modes of action/primary targets if successful. The supply of the ca.  $10^6$  new chemical structures required for this approach is from the equally new technology of combinatorial chemistry.

## II. CHLOROPHYLL BIOSYNTHESIS AND ELECTRON FLOW

### A. Inhibition of Protoporphyrinogen Oxidase

The development of the structurally different protoporphyrinogen oxidase–(PPO-) inhibiting herbicides has shown a trend of increasing herbicide potency and therefore decreasing application rates over the last ca. 30 years. Whereas  $2\text{--}3\text{ kg ha}^{-1}$  was required for some of the earlier nitrodiphenylethers, only  $30\text{--}60\text{ g ha}^{-1}$  is sufficient for the phenylpyrazoles and phthalimides (Fig. 1). Among the diphenylethers, most compounds contain a nitro group. However, compounds with chlorine instead of nitro are also herbicidally active (17). The ortho position to the nitro group of active compounds is very variable and may contain *O*-alkyl, carboxylic acids, carboxylic esters and amides, amines, or hydrogen. Newer chemical groups of PPO herbicides show an even greater variety. A 1998 survey of the diverse structures has been published (18). A sampling of PPO inhibitor herbicides

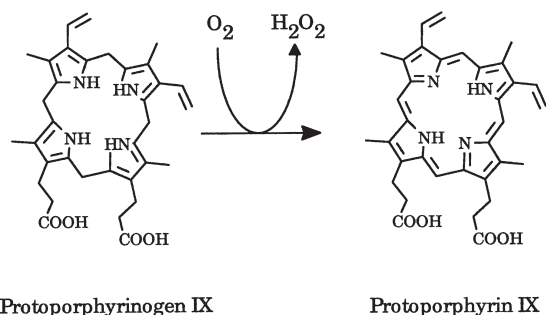


**Figure 1** Structures of herbicides inhibiting the chlorophyll biosynthesis enzyme protoporphyrinogen oxidase. Examples from five different structural groups are provided, out of a total of ca. 40 commercial herbicides that inhibit at this target.

is provided in Fig. 1. The PPO herbicides primarily affect dicotyledonous species, but some gramineous weeds are also controlled.

### 1. Primary Target: An Inhibition Paradox

The enzyme protoporphyrinogen oxidase (PPO) is part of a multienzyme complex for the synthesis of tetrapyrrole structures (chlorophylls,



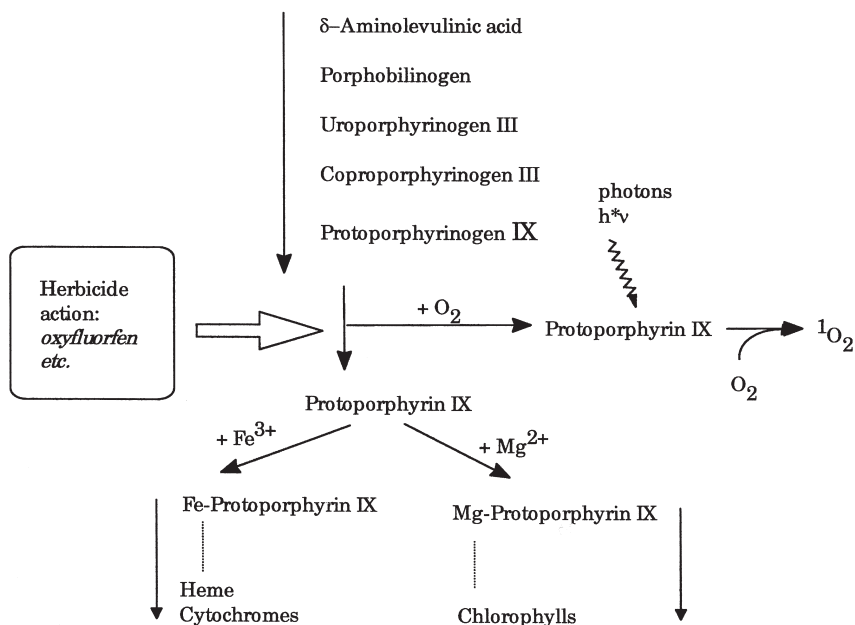
**Figure 2** The enzyme protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX with molecular oxygen, generating hydrogen peroxide.

cytochromes, and heme) (Fig. 2). The biochemical function of PPO is shown in Fig. 3. Surprisingly, in the presence of herbicides that inhibit PPO, accumulation of the product of the inhibited reaction, protoporphyrin IX (proto. IX), occurs. Obviously, when PPO is inhibited, the substrate protoporphyrinogen IX is set free of the multienzyme complex and may then react spontaneously with free molecular oxygen, generating the product proto. IX without participation of the enzyme. Alternatively, protoporphyrinogen IX may be oxidized by an herbicide-resistant oxidase (19, 20). The enzyme may be tested *in vitro* and is then inhibited by the herbicides at micromolar ( $\mu\text{M}$ ) (early herbicides) to nanomolar ( $\text{nM}$ ) (later developments) concentrations (17, 20–22). The great variety of herbicides inhibiting protoporphyrinogen oxidase is explained by the fact that their structures mimic part of the substrate molecule. The inhibitor structures with their two rings, side chains, and frequent carboxy groups may be superimposed on part of the three-dimensional structure of the true substrate, protoporphyrinogen IX (17, 23).

## 2. Affected Metabolism: Loss of Regulation

Free proto. IX generated by the spontaneous oxidation of protoporphyrinogen IX cannot react with one of the chelatasases, which accept only proto. IX within the multienzyme complex to form either the Fe or the Mg chelates. Because of the loss of tetrapyrrole precursors, in the presence of herbicides, the synthesis of chlorophylls via proto. IX is no longer properly regulated. Protoporphyrin IX is therefore synthesized in high amounts. In the dark, proto. IX can accumulate in the tissue, but in the light ( $h\nu$ ) it reacts as a photosensitizer and transfers its excitation energy to molecular oxygen. The resulting highly reactive singlet oxygen ( $^1\text{O}_2$ ) reacts with





**Figure 3** Pathway of tetrapyrrole synthesis in plants. The two branches employing either iron or magnesium chelataes are indicated. The herbicide action on protoporphyrinogen oxidase leads to liberation of the substrate from the multi-enzyme complex and its spontaneous oxidation to protoporphyrin IX. Protoporphyrin IX in turn acts as a photosensitizer and transfers its excitation energy to oxygen, generating the highly reactive singlet oxygen.

unsaturated membrane lipids and starts peroxidative chain reactions (see Sec. II.D). Finally, membranes and other cell constituents are oxidatively destroyed.

### 3. Physiological Effects: Consequences of Photosensitization

The mechanism of action of PPO inhibitor herbicides is basically that of excessive amounts of activated singlet oxygen.

1. After only 1–3 h, photosynthetic  $CO_2$  fixation ceases and membrane damage may be seen in electron micrographs. Chloroplasts swell, whereas endomembranes (plasmalemma, tonoplast) lose contrast.
2. The loss of turgor and the leakage of soluble cellular compounds into the apoplast are among the next consequences (2–20 h).

3. Chloroplasts and mitochondria are both directly affected since both contain PPO. However, because the maximal rates of synthesis for chlorophylls in the chloroplasts and for cytochromes in mitochondria differ by at least two orders of magnitude, the damage to chloroplasts is of paramount importance for leaves. In other tissue or in the dark, damage develops much more slowly and to a lesser extent.
4. With increasing time, lipophilic vesicles accumulate in the cytoplasm, and ethane, pentane, and malondialdehyde are developed and accumulated as products of membrane lipid destruction (2–20 h).
5. The photosynthetic pigments are gradually destroyed: carotenoids, which may quench singlet oxygen to some extent, are first destroyed when overloaded; chlorophylls, which are basically protected by carotenoids and  $\alpha$ -tocopherol (see Sec. III.A.1), are destroyed later (after 1–2 days).
6. The production of ethylene is a stress response involving the synthesis of biosynthetic enzymes, e.g., 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. The massive ethylene amounts may then lead to leaf abscission.
7. The presence of sublethal amounts of singlet oxygen over several days may induce other stress responses: the induction of phenylalanine ammonia lyase (PAL) and other phenylpropanoid/isoflavonoid pathway enzymes results in the synthesis of phytoalexins (e.g., hemigossypol, glyceollin, phaseollin, pisatin).
8. Singlet oxygen may also be produced by the plant after fungal or pathogen attack and then serve as a line of defense; PPO inhibitor herbicides may under certain conditions interfere with these systems.

#### 4. Visible Symptoms: Rapid Desiccation

As may already be concluded from the descriptions given, PPO inhibitor herbicides act rapidly in full sunlight. Pretreatment in the dark, followed by strong light, leads to very rapid phytotoxicity (within ca. 1–2 h), because proto. IX accumulates during the dark phase. After “normal” plant treatment in the seedling stage, the phytotoxicity develops within a few hours to a few days. The molecular mechanisms of oxidative damage in the tissue are described elsewhere (see Sec. II.D). Visible symptoms in leaves include wilting, necrosis, and browning of the tissue after rapid action, or light green coloring to chlorosis after slow action.

## B. Inhibition of Photosystem II

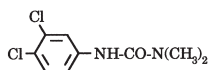
Of the herbicides shown in Fig. 4, ureas form the largest group, in which ca. 40 compounds have been commercialized. The second largest is the triazine group with ca. 25 compounds. The urea compound diuron is well known in plant physiological research as 3-(3,4 dichlorophenyl)-1,1-dimethyl urea (DCMU). For more than 40 years, it has served as the standard inhibitor of photosynthetic electron flow in botanical laboratories around the world. It is still used for nonselective (total) vegetation control. Fluometuron also inhibits carotenoid biosynthesis, in addition to photosystem II (PS II), presumably at the target phytoene desaturase (see Sec. III.A.6). Among the triazines, the names of chloro compounds end with *-azine*, those with *S*-methyl end with *-tryne*, and those with *O*-methyl end with *-meton* (2).

The great diversity of structures that inhibit at PS II reflects a large quinone binding niche of the target protein, which can adapt many different molecules. During binding, a sensitive central binding area is important for inhibition, whereas other peripheral binding contributions are important for binding specificity but not for inhibition. This early concept of different binding areas was termed *subreceptors*. The structural element  $-C(=O)-NH-$  or its electronic similarities were recognized as required for the central binding element. A binding of ligands not leading to inhibition has, however, not been observed. The situation described here for the herbicidal inhibitors of PS II is similar to that for the herbicidal inhibitors of ALS, which apparently also bind into a quinone binding niche and show a similarly great variety of inhibitory structures (see Sec. IV.B). Also, the situation for resistance mutations described for the two primary targets is very similar.

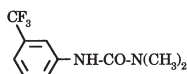
### 1. Primary Target: The Plastoquinone Binding Niche

The structures of the higher plant PS II reaction center proteins D1 and D2 were originally modeled by analogy to the known crystal structures of the bacterial photosynthetic reaction center proteins, called *L* and *M* (24, 25). In 2001, an entire cyanobacterial PS II crystal structure was obtained (26). The D1 and D2 chains form a dimer with six chlorophyll *a*, two pheophytin, and one plastoquinone at the  $Q_A$  site and nonheme iron, all bound to the protein and required for the initial photochemical process (Figs. 5 and 6). The  $Q_B$  site is accessible to the mobile plastoquinone pool. Both the D1 and D2 proteins span the chloroplast thylakoid membrane five times (helices A to E). In addition, the membrane spanning helices C, D, and E are connected by  $\alpha$ -helices that lie perpendicular to the membrane face on the inner (helix CD) and outer (helix DE) sides, respectively. Loops connect the  $\alpha$ -helices. Figure 5 shows the plastoquinone binding region of the D1 protein.

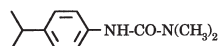
## Ureas



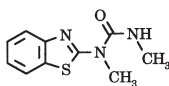
Diuron



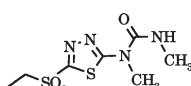
Fluometuron



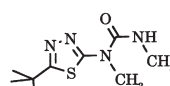
Isoproturon



Methabenzthiazuron

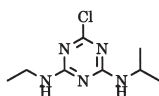


Ethidimuron

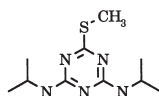


Tebuthiuron

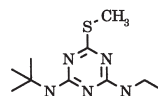
## Triazines



Atrazine

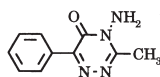


Prometryne

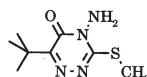


Terbutryne

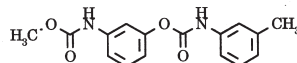
## Triazinones and Phenylcarbamates



Metamitron

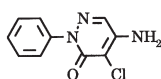


Metribuzin

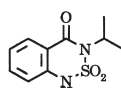


Phenmedipham

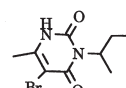
## Miscellaneous



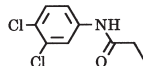
Pyrazon / Chloridazon



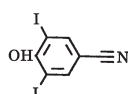
Bentazon



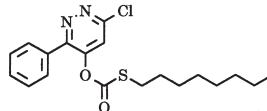
Bromacil



Propanil



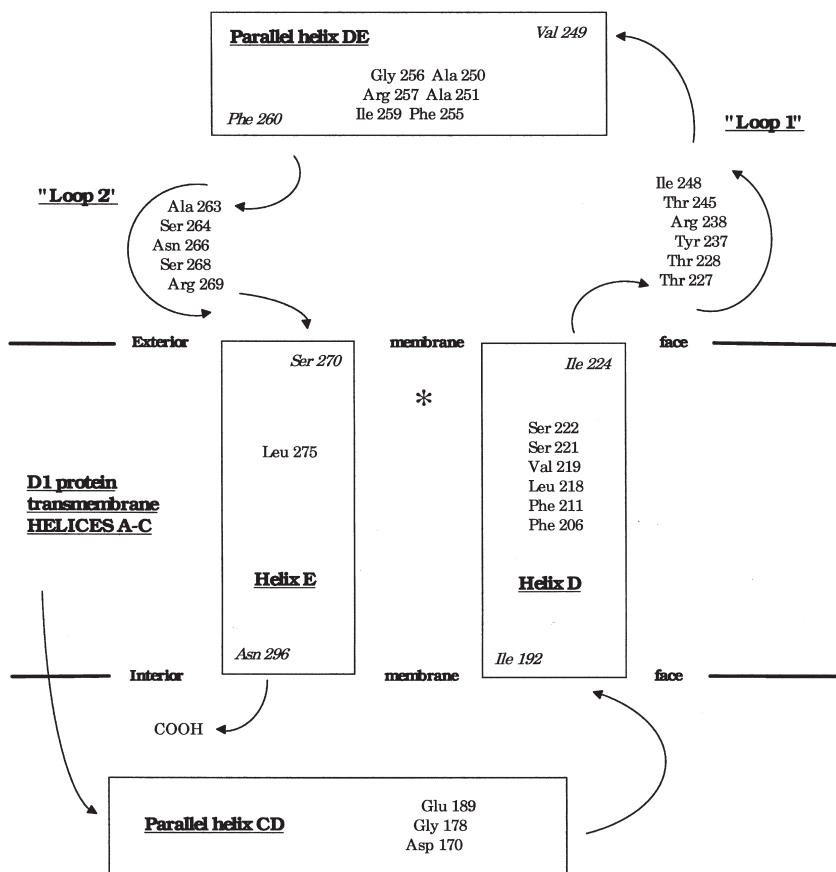
Ioxynil



Pyridate

**Figure 4** Of the ca. 100 commercial herbicides known to inhibit at photosystem II, a small selection from the main structure groups is presented here. Most of these herbicides have been known for more than 20–30 years.

Since PS II inhibitor herbicides bind competitively to the plastoquinone  $Q_B$  binding site, this is also the binding site for the herbicides. Figure 5 also takes advantage of the fact that the herbicide binding region can be recognized by the resistance mutations that cluster in the vicinity of the



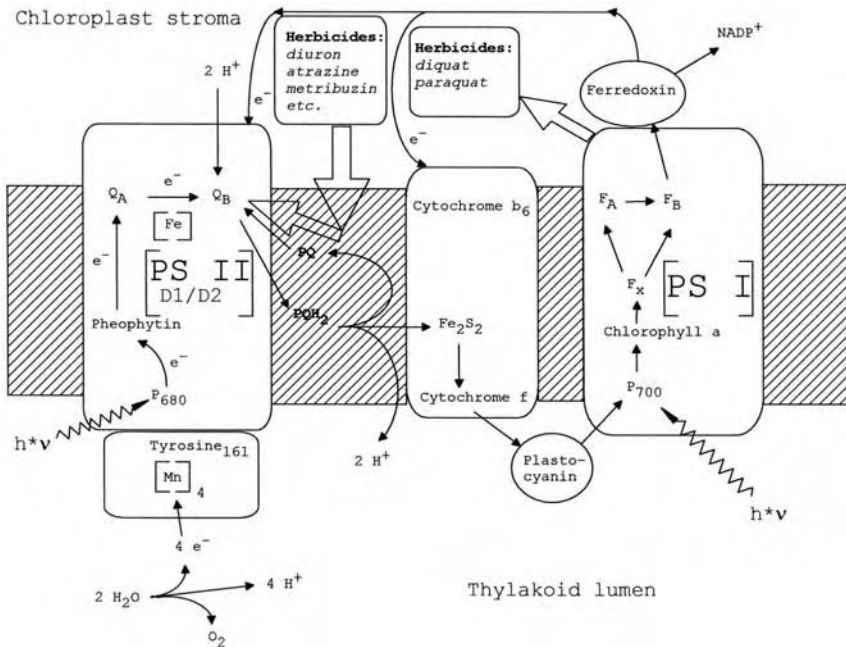
**Figure 5** The selection of amino acids presented here within the structural elements of the D1 protein is restricted to those that have led to herbicide-resistant photosynthesis when altered by mutation. The individual positions are located in two transmembrane and two parallel  $\alpha$ -helices and also in the loops connecting these. They cluster around the plastoquinone  $Q_B$  binding site, which is located between helices D and E close to the exterior membrane face, indicated here by an asterisk (25–27). Additionally, amino acids located at the ends of the  $\alpha$ -helices D, DE, and E are given in *italic*. These are not resistance locations. Other amino acids are not presented. It is evident that the mutation sites “titrate” the surrounding of the herbicide binding niche. Serine 264 is changed to glycine in the well-known triazine resistance cases, which occurred in many weeds under agricultural conditions (2, 12). The other resistance locations have been found in the green alga *Chlamydomonas reinhardtii* and in cyanobacteria, mainly *Synechocystis* sp. Also, *Euglena* sp. and higher plant cell cultures were used for the selection of resistant mutants (28). Compare with Fig. 6. (Adapted from Ref. 30.)

binding niche. In the three-dimensional (3D) structure of the D1 protein, helices D and E are tilted, and the carbonyl groups of the plastoquinone bound to the Q<sub>B</sub> site point to serine 264 in “loop 2” and to histidine 215 in helix D (not shown in Fig. 5). The phytol side chain of plastoquinone bound to the Q<sub>B</sub> site may extend into the lipophilic membrane interior.

Herbicides binding in the PQ binding niche may find many different orientations, generally filling only part of the total available space. In the 3D structure, the binding to and the interference with individual amino acids of the binding niche can be reconstructed for individual herbicides (25, 27). Interestingly, in resistant PS II centers, other PS II inhibitor herbicides not used in the selection process either may require similarly elevated concentrations in order to obtain inhibition (cross-resistance), may not be affected at all, or may be inhibitory at lower concentrations than required in the wild type (supersensitivity) (28, 29). In terms of the commonly used pI<sub>50</sub> value (negative decadic logarithm of the molar concentration required for 50% inhibition, usually between 5 and 7 when measured in isolated chloroplasts) this means that the pI<sub>50</sub> is strongly lowered, unchanged, or increased. In many cases, compounds from the same inhibitor group (e.g., ureas, triazines, uracils) show similar cross-resistance patterns. However, exceptions to this rule occur. The triazine-resistant plants are, e.g., nearly unchanged in their sensitivity to diuron. For a current list of herbicide-resistant plants see Ref. 12. In Ser264 mutated plants the electron transport is slowed somewhat, leading to the so called yield penalty of ca. 20% in crop plants carrying this mutation (e.g., in Canola rape).

## 2. Affected Metabolism: Blocking the Photosynthetic Electron Flow

Figure 6 shows a scheme for the electron flow in and among the three integral chloroplast thylakoid membrane protein complexes PS II, photosystem I (PS I) and the cytochrome b<sub>6</sub>/f complex. *Integral* means that most of the protein subunits contain several hydrophobic spans crossing the membrane. Cofactors are bound to these protein subunits as prosthetic groups. At their interfaces with either the thylakoid lumen or the chloroplast stroma they interact with different electron carriers, presented here by arrows showing the electron flow from water to oxidized nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) in a zigzag way across the membrane. Each of these protein complexes contains a number of individual protein subunits binding pigments and/or fulfilling different functions (31–33). Of prime importance for the two modes of herbicide action realized here (open arrows in Figure 6) are (a) access to the PQ binding niche of the D1 protein of PS II from within the lipid membrane



**Figure 6** Vectorial electron flow in chloroplast thylakoid membranes from water to oxidized nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>). The three membrane spanning proteins not only contain the pigments and cofactors for electron transport but also carry protons into the chloroplast interior, generating the proton gradient required for energy conservation (adenosine triphosphate [ATP] synthesis). Two types of herbicide interference are shown: either by herbicide diffusion into the lipid membrane interior and replacing plastoquinone (PQ) at its binding site (Q<sub>B</sub>) or by the herbicide acceptance of low potential electrons from photosystem I at the ferredoxin reduction site.

phase (diuron, etc.) and (b) electron transfer to paraquat, etc., at PS I. Photons absorbed by chlorophylls of the light-harvesting chlorophyll protein complexes (LHCP complex, the fourth integral membrane protein, not represented here) travel to the special pigment P<sub>680</sub> and, after excitation and charge separation between P<sub>680</sub> and pheophytin, lead to electron flow to the bound plastoquinone<sub>A</sub> (Q<sub>A</sub>). The lost electrons are replaced from the tetranuclear Mn complex via tyrosine 161 and eventually by the oxidation of water to molecular oxygen and the simultaneous liberation of protons (H<sup>+</sup>) in the thylakoid lumen. The protons accumulating in the interior either from water splitting or by import from the chloroplast lumen in the plastoquinone redox cycle around the cytochrome b<sub>6</sub>/f complex lead to a pH gradient that is the driving force for adenosine triphosphate (ATP)

synthesis through ATP synthase (see Sec. II.E), the fifth integral membrane protein complex (also not included here).

The PS II inhibitor herbicides can be classified as plastoquinone analogs since they replace the mobile plastoquinone  $Q_B$  in its binding niche (27). Therefore,  $Q_B$  is no longer reduced and the bound  $Q_AH_2$  is not oxidized. Two consequences are (a) the immediate stopping of  $CO_2$  fixation because the mobile redox carrier  $NADP^+$  is no longer reduced and (b) an accumulation of excitation energy at  $P_{680}$ . Whereas (a) has little phytotoxic potential for at least several hours in the light (b) leads to a transfer of excitation energy from  $P_{680}$  and its triplet state to molecular oxygen and the formation of the highly reactive singlet oxygen ( $^1O_2$ ), comparable to the inhibitors of protoporphyrinogen oxidase (see Sec. II.A). Here, however,  $^1O_2$  is generated in the extreme vicinity of the PS II reaction center and seems to react directly with the D1 protein. The resulting PS II damage, called *photodamage* and leading to *photoinhibition* is normally neutralized by a repair cycle that has an important function for the productivity of photosynthesis (34). In the presence of herbicides binding to the D1 protein, however, this repair cycle is severely affected and the membranes and tissues are destroyed (see Sec. II.D).

### 3. Physiological Effects: Overloading the Repair Systems

The  $^1O_2$  produced when the electron transfer to PQ is inhibited has effects similar to those occurring after inhibition of protoporphyrinogen oxidase, as described previously. However, since the rate of  $^1O_2$  production is lower and differently localized, symptoms and effects occur more slowly and may also be different:

1.  $CO_2$  fixation is inhibited rapidly, usually after a few hours in full sunlight.
2. More specifically, herbicides bound to the  $Q_B$  binding site inhibit the rapid turnover and replacement of the D1 reaction center protein after photodamage has occurred, leading to irreversible damage.
3. Nitrite accumulates because reduced ferredoxin for nitrite reductase is no longer available.
4. Ascorbate becomes peroxidized and  $\beta$ -carotene is destroyed, starting immediately after inhibition and progressively leading to 90% loss after 1–2 days.
5. Chlorophylls become oxidized with some delay after their protection by  $\beta$ -carotene and other molecules such as  $\alpha$ -tocopherol has been lost.



6. The electron transport system as measured by artificial donors and acceptors is destroyed after 1–2 days. However, the water splitting part is lost after only a few hours.
7. After about 20 h, the products of membrane fatty acid peroxidation, ethane and malon dialdehyde, can be detected.

#### 4. Visible Symptoms: Bleaching Details

In full sunlight and at 100% inhibition of electron flow, leaf tissue becomes wilted after a few hours and desiccates to dark brown after a few days. However, when the inhibition develops more slowly and full sunlight is not available, the leaf tissue turns light green to yellow and eventually becomes white and/or necrotic. This description is valid after postemergence application.

After preemergence application and root uptake of the herbicide, the appearance of treated leaves may be quite different, depending on water uptake, humidity, and water solubility of the herbicide. In principle, herbicides with low water solubility (w.s.) (1–20 ppm) move slowly and do not distribute evenly in the leaf, often leading to leaves with white assimilatory tissue but green veins. On the contrary, herbicides with high w.s. (>1000 ppm) move rapidly with the transpiration stream, accumulating in the leaves and resulting in even distribution of toxicity symptoms.

Moreover, the potency of the individual herbicides differs greatly and is strongly modulated by the w.s. The common procedure of measuring herbicide activity by  $pI_{50}$  values (negative  $\log_{10}$  of the concentration required for 50% inhibition) in isolated chloroplasts gives a value containing the influences of (a) the lipophilic distribution between membrane and aqueous phase and (b) the binding constant in the binding niche proper. For example, a herbicide such as metamilon that has a w.s. of 1820 mg l<sup>-1</sup> and a  $pI_{50}$  of 5.4 requires a field application rate of 3–4 kg ha<sup>-1</sup>. It is a good herbicide because of its superior selectivity in sugar beet. On the other hand, metribuzin with a largely similar w.s. of 1200 mg l<sup>-1</sup> but with a  $pI_{50}$  of 6.7 is two orders of magnitude more active/specific at the binding site. The field application rate of metribuzin is accordingly only 125–500 g ha<sup>-1</sup>. The symptoms are those of an even distribution, as described previously.

When a herbicide does not kill the treated plant, it may still act at sublethal rates and partially inhibit photosynthesis for several hours to days. This is often the case in treated crop plants, which survive the herbicide treatment because they can rapidly detoxify the active compound. Still, when partial inhibition of photosynthesis occurs over several days, the plants react by a “shade adaptation” i.e., the plants obviously sense the low

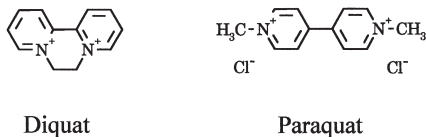
photosynthetic productivity as if they grew in shade condition. The leaf metabolism and the chloroplast ultrastructure and biochemical composition become adapted to low light intensities (35, 36). This adaptation can be seen as the “greening effect” frequently observed in treated crop plants in early spring. It includes a chloroplast restructuring (more grana lamellae) and an intensified nitrogen metabolism (higher nitrate reduction rates and more soluble protein).

### C. Photosystem I Electron Diversion

Compounds with a standard redox potential between  $-300$  and  $-700$  mV and a rapid spontaneous reoxidation by molecular oxygen (auto-oxidation) may be used as herbicides at the reducing side of photosystem I (PS I). Also, the radical must be stable long enough to diffuse far enough to be able to react with  $O_2$ . In spite of tremendous efforts and the testing of many compounds that fulfill these conditions, only paraquat (Fig. 7) has gained commercial importance (37). In plant physiology research, paraquat has had a second “career” under the name *methylviologen* (MV). Diquat and paraquat act exclusively as postemergence herbicides and are used for rapid desiccation of green vegetation before mechanical harvest, e.g., in potatoes, or for total vegetation control in orchards and vineyards. Diquat is also used for aquatic weed control. Both compounds have high mammalian toxicity but adsorb strongly to soil particles and degrade quickly in the environment.

#### 1. Primary Target: Negative Potential Electron Misuse

As can be seen in the scheme of zigzag photosynthetic electron flow in thylakoid membranes in Sec. II.B on PS II inhibitors (Fig. 6), low-potential electrons (at least  $-450$  mV) supplied by photosystem I (PS I) normally used to reduce ferredoxin (38) can directly reduce diquat or paraquat at the lumen side of thylakoid membranes. These redox herbicides can accept only



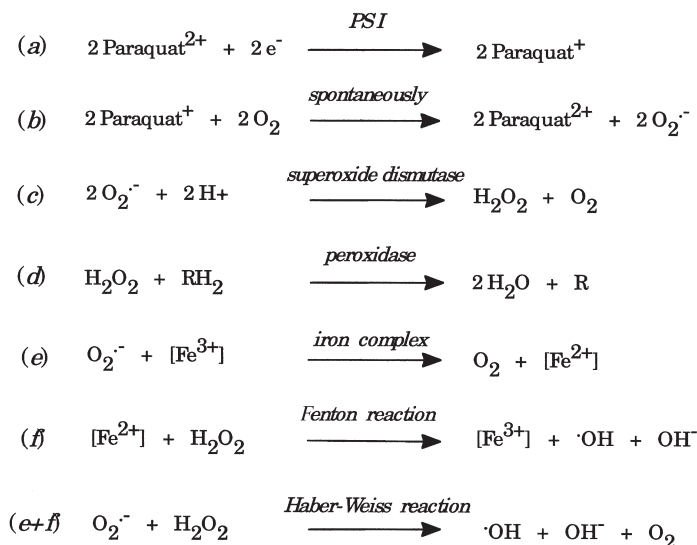
**Figure 7** Structures of herbicides accepting electrons from photosystem I. One of the two quaternary nitrogen atoms becomes reduced, rendering the divalent cations monovalent in the reduced form. Only one electron can be accepted per molecule.

one electron, which they spontaneously transfer to molecular oxygen to yield the superoxide anion ( $O_2^{\bullet-}$ ). In principle,  $O_2^{\bullet-}$  is the molecular species that, when produced in massive amounts, mediates the damage that occurs after treatment with these PS I electron acceptor herbicides.

## 2. Affected Metabolism and Physiological Effects: The Superoxide Overload

After physiological generation of  $O_2^{\bullet-}$ , the normal pathways of dissipation of the inherent oxidizing and reducing power of  $O_2^{\bullet-}$  become effective. In the reactions in Fig. 8a and b,  $O_2^{\bullet-}$  normally reacts with a “stromal factor” instead of paraquat; also a direct reduction of  $O_2$  at the reducing side of PS I appears possible (39). Low amounts of  $O_2^{\bullet-}$  are dealt with effectively by the enzymes superoxide dismutase (Fig. 8c) and peroxidase (Fig. 8d, ascorbate for  $RH_2$ , Sec. II.D). Only when these protective mechanisms are overloaded do lipid peroxidation and tissue damage occur.

In the presence of paraquat and strong light, high rates of  $O_2^{\bullet-}$  synthesis occur (Fig. 8a and b). However, neither the  $O_2^{\bullet-}$  anion nor the  $H_2O_2$  has sufficient reactivity for the strong and rapid herbicidal action seen



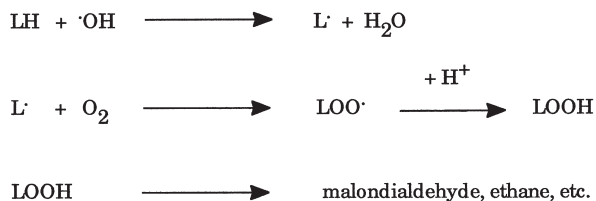
**Figure 8** Pathway of electrons from reduced paraquat (which is a monovalent cation as a result of the presence of two quaternary nitrogens in paraquat!) to the final toxicant, the hydroxyl radical ( $\cdot OH$ ).

after paraquat treatment. Only the hydroxyl radical can serve as a mediator of this rapid herbicidal action. In healthy tissue the strategy appears to be the avoidance of the production of the hydroxyl radical ( $\cdot\text{OH}$ ). This is achieved by sequestration and protein binding of ferrous ions. In case some damage has already occurred after paraquat treatment,  $\text{Fe}^{3+}$  becomes accessible and can accept electrons from  $\text{O}_2^{\cdot-}$  (Fig. 8e). The next step is the immediate reduction of hydrogen peroxide to yield the hydroxyl radical (Fig. 8f). Both reactions taken together constitute the Haber–Weiss reaction, which is in essence a metal-catalyzed reduction of hydrogen peroxide to  $\cdot\text{OH}$  and  $\text{OH}^-$ . The hydroxyl radical has a redox potential of ca. +2 V and is highly reactive. It probably does not occur as a free species but forms complexes with metals and  $\text{H}_2\text{O}_2$  or with  $\text{H}_2\text{O}_2$  and reduced paraquat, which carries one positive charge.

The  $\cdot\text{OH}$  or its complexed forms easily abstract hydrogens from unsaturated lipids such as linolenic and linoleic acids with the formation of water. The oxidized lipids then react with oxygen, forming lipid peroxides (Fig. 9). Also, ascorbate, glutathione,  $\beta$ -carotene, and  $\alpha$ -tocopherol are progressively oxidized. Some of these reactions and protective systems are further described elsewhere (see Sec. II.D).

### 3. Visible Symptoms: Very Rapid Desiccation

The very rapid production of singlet oxygen in paraquat-treated photosynthetic tissue in strong light can lead to the first visible symptoms within 10 minutes. These symptoms are wilting and desiccation of the tissue. They become manifest within a few hours. No time is available for complete chlorophyll breakdown, because the membranes are destroyed very rapidly, and the tissue then desiccates while still green. The rapid action in the light also means that no herbicide is translocated out of a treated leaf under these conditions.

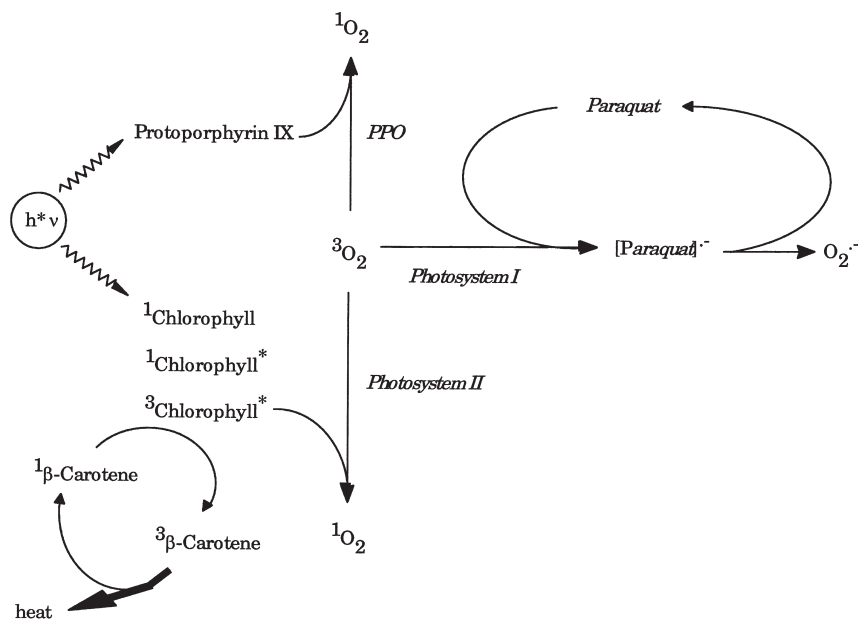


**Figure 9** Lipid (LH) peroxidation induced by the hydroxy radical. The lipid radical ( $\text{L}^\cdot$ ) produced by hydrogen abstraction can induce radical chain reactions or react with oxygen to form labile peroxides (compare with Fig. 12).

In the dark, paraquat is translocated in the phloem throughout the entire plant, with an enhanced herbicidal action after exposure to light. In nonphotosynthetic tissue, similar lipid peroxidations occur, but at a much slower rate. A microsomal cytochrome P450 monooxygenase may be one of the enzymes that reduce paraquat under these conditions.

#### D. Photo-oxidative and Protective Pathways

Several of the primary herbicidal targets present in chloroplasts lead to oxygen activation and tissue damage as a result of the activated oxygen species generated. Four mechanisms of oxygen activation are provided in a common scheme (Fig. 10).

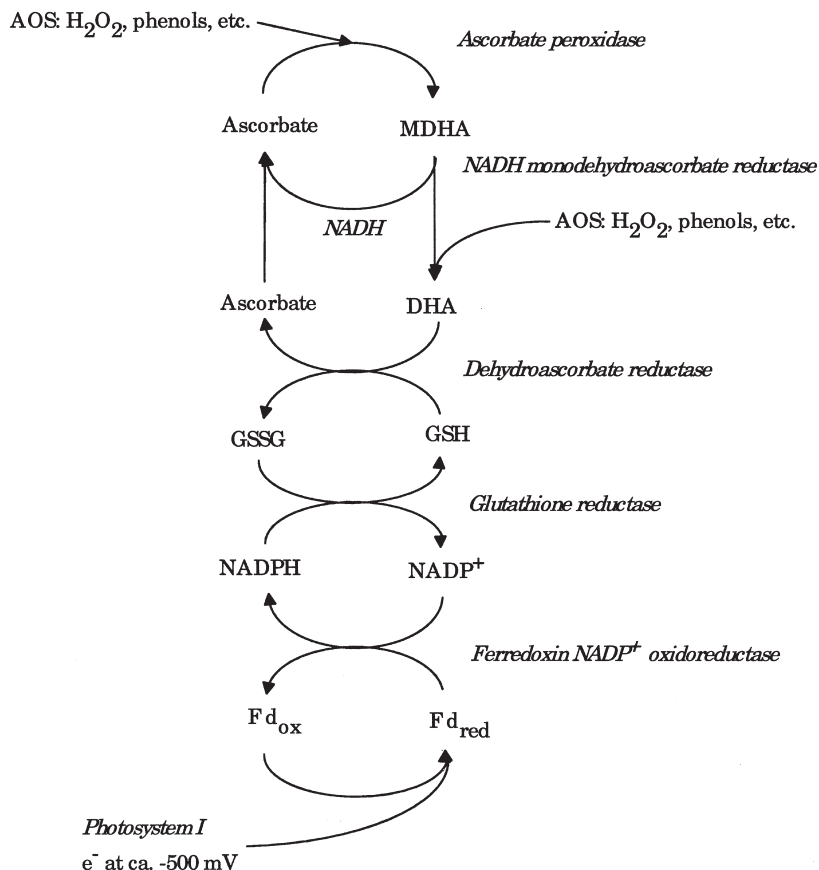


**Figure 10** Pathways of light-dependent oxygen activation in herbicide-treated plants. Ground state triplet oxygen either may be turned into the activated singlet state by photosensitization via protoporphyrin IX (PPO inhibitors, Sec. II.A) or via excited triplet chlorophyll (photosystem II inhibitors, Sec. II.B) or may be reduced to the superoxide anion radical (photosystem I electron acceptors, Sec. II.C). Inhibitors of  $\beta$ -carotene synthesis (Sec. III) also lead to singlet oxygen production because excess excited triplet chlorophyll stages can no longer be quenched by the production of triplet  $\beta$ -carotene.

Of the four mechanisms given, three are primary herbicidal targets, here presented in italics, whereas  $\beta$ -carotene is the product of a synthetic sequence that contains at least seven primary herbicidal targets (see Sec. III). All four mechanisms serve different aspects of photosynthesis. Three lead to the activation of ground state triplet oxygen to the highly reactive singlet state oxygen ( $^1\text{O}_2$ ), and one leads to reduction of oxygen to the superoxide anion radical ( $\text{O}_2^{\bullet-}$ ):

1. Protoporphyrin IX, which accumulates after inhibition of the chlorophyll biosynthetic enzyme protoporphyrinogen oxidase (PPO, Sec. II.A), serves as a photosensitizer.
2. Inhibition of electron flow through photosystem II (Sec. II.B) leads to the accumulation of excess excitation energy in the triplet state chlorophyll, which reacts with ground state triplet oxygen to form excited  $^1\text{O}_2$ .
3. Singlet ground state  $\beta$ -carotene in the photosystem II complex serves as a quencher for activated triplet state chlorophyll, thereby being itself activated to its excited triplet state. Triplet state  $\beta$ -carotene converts spontaneously into the ground state and emits the energy as heat. Also zeaxanthin, which is generated by the xanthophyll cycle, contributes to the thermal energy dissipation (40). Herbicides inhibiting carotenoid biosynthesis (see Sec. III) therefore also kill tissue by the generation of excess amounts of  $^1\text{O}_2$ .
4. The herbicide target acting through oxygen reduction is photosystem I. Here, electrons are accepted by an auto-oxidizable acceptor (paraquat in this example) from photosystem I and generate the superoxide anion radical ( $\text{O}_2^{\bullet-}$ , Sec. II.C).
5. Mitochondrial electron transport can lead to the production of reactive oxygen species through the non-proton-pumping reduced nicotinamide adenine dinucleotide phosphate dehydrogenases accepting either NADPH or NADH (41).

The sequence of enzymes to deal with activated oxygen species (AOS) is constituted by superoxide dismutase (SOD), ascorbate peroxidase (APX), NADH monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and ferredoxin NADP<sup>+</sup> oxidoreductase (FNR). Of these, SOD reacts with  $\text{O}_2^{\bullet-}$  to produce the AOS  $\text{H}_2\text{O}_2$ . The other five enzymes are shown in Fig. 11. Further, ascorbate is oxidized by AOS and  $\alpha$ -tocopherol, while other radical scavengers stop radical chain reactions started by events such as unsaturated fatty acid peroxidation from singlet oxygen. Chloroplasts contain ascorbate and

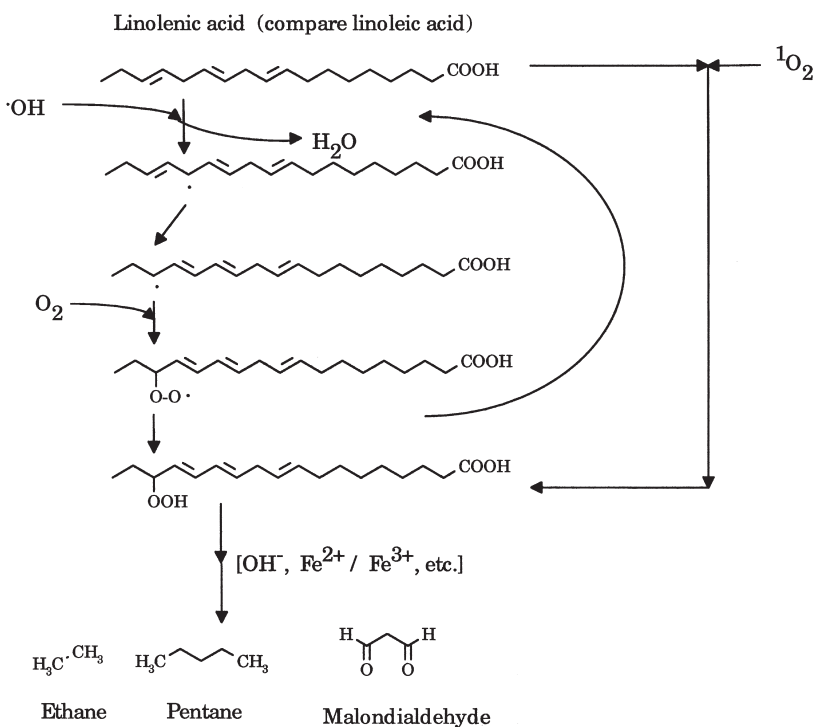


**Figure 11** The chain of reactions that destroys highly reactive molecules and AOS that have been produced in the course of herbicidal action. Herbicides that inhibit photosystem II or divert electrons from photosystem I inhibit the reduction of ferredoxin and thereby indirectly also inhibit the chain of reactions shown here. AOS, activated oxygen species; MDHA, monodehydroascorbate; DHA, dehydroascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; Fd, ferredoxin.

reduced glutathione in high concentrations up to 25 mM. They are therefore well equipped to reduce and inactivate the AOS that are synthesized naturally in strong light or under natural stress conditions (39, 42–44). The necessary reducing power for the regeneration of reduced glutathione and ascorbate eventually arises from the photosynthetic electron transport chain via reduced ferredoxin and NADPH. In the presence of herbicides

generating high amounts of  $^1\text{O}_2$  or  $\text{O}_2^{\cdot-}$  these protective systems are overloaded, and AOS can accumulate and generate herbicidal damage. In the presence of inhibitors of PS II,  $\text{NADP}^+$  reduction is obviously stopped, but the photosynthetic electron transport chain is also rapidly inactivated by the other herbicides that cause oxidative damage.

As mentioned, unsaturated lipids are peroxidized by  $^1\text{O}_2$  or by the hydroxy radical and/or its complexed forms. Direct addition of  $^1\text{O}_2$  or electron abstraction by the hydroxy radical (see Secs. III and II.C) leads to peroxidation. The chloroplast unsaturated fatty acids linoleic and linolenic acid are then further oxidized to yield compounds such as malondialdehyde and ethane. Linolenic acid produces ethane and linoleic acid produces pentane (Fig. 12). Malondialdehyde is an end product of repeated oxidation at similar positions. The reactions in a plant cell undergoing photo-oxidative



**Figure 12** Peroxidation of the unsaturated chloroplast linoleic and linolenic fatty acids. More and other (per)oxidation products are produced by the participation of, e.g., metal and acid-base catalysis.



damage are certainly much more diverse and also include other reactants such as metal catalysis.

### E. Uncoupling of Oxidative Phosphorylation and Photophosphorylation

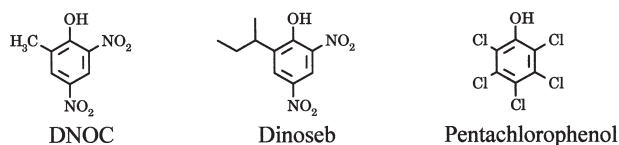
The structures of compounds acting as uncouplers (Fig. 13) are either dinitrophenols, hydroxynitriles, or other compounds that can dissociate a proton at physiological pH. The hydroxynitriles are also photosystem II inhibitors and are represented by ioxynil in Fig. 4.

#### 1. Primary Target: The Transmembrane pH Gradient

Adenosine triphosphate synthase was introduced in Sec. II.B as the fifth integral thylakoid membrane protein complex. Similarly, an ATP synthase is present in the inner mitochondrial membranes. The zigzag vectorial electron transport produces a pH gradient across both thylakoid and mitochondrial membranes. Thereby, the pH becomes more acid inside thylakoids, and similarly in the mitochondrial intermembrane and cristae space. After organelle disruption and subsequent vesicle formation, thylakoids still pump electrons into their interior space, whereas the mitochondrial vesicles formed during membrane healing are inside-out and therefore become more alkaline inside during vectorial electron transport.

The chemiosmotic theory explains ATP synthesis by the buildup and use of a proton gradient as the energy supply for ATP synthase ( $\Delta$  pH between 1.4 in mitochondria and 3.5 in chloroplast thylakoids). The stoichiometry is three electrons transported back per ATP synthesized. The ATP synthase is composed of an integral proton transport channel  $CF_0$  and a lumen-exposed  $CF_1$  part of five different protein subunits. As indicated,  $CF_1$  is located inside the mitochondrial vesicles formed after disruption.

The uncoupling herbicides shown in Fig. 13 are weak acids, which can transport protons through membranes, depending on the pH gradient and



**Figure 13** Structures of some herbicidal phenols that are known to degrade the transmembrane proton gradient and therefore uncouple phosphorylation.

their ability to dissolve into and penetrate the lipophilic membrane interior. They are also called *protonophores*. Similar proton transport is caused by ioxynil and similar nitriles, which are D1 inhibitors (see Sec. II.B). Conversely, the phenols in Fig. 13 are also inhibitors of photosynthetic electron transport. However, this inhibition is of only minor importance for herbicidal action. The process of proton transport leading to a breakdown of the pH gradient and loss of ATP synthesis is known as *uncoupling*.

Another group of herbicides with uncoupling activity, irrespective of their normal primary target, are lipophilic herbicides that can accumulate in membranes. Trifluralin (also perfluidone and some lipophilic diphenylethers and biscarbamates) at high dosage accumulates in target organelle membranes at concentrations up to 10–100  $\mu\text{M}$ . These high concentrations induce “lipid perturbations” in the membrane interior, which allow the leakage of protons along the pH gradient, therefore by definition uncoupling ATP synthesis.

## 2. Affected Metabolism and Physiological Effects:

### Loss of Energy Supply

The loss of oxidative phosphorylation and/or photophosphorylation dramatically affects the anabolic processes that maintain cellular integrity. Uncoupling of photophosphorylation by PS II inhibitors is, however, of only minor practical importance because the inhibition of electron transport is more sensitive, thereby producing most of the herbicidal effects. In contrast, the uncoupling activity of the phenol compounds in mitochondria is commonly obtained at micromolar concentrations, whereas 10 times higher concentrations would be required for the inhibition of oxidative mitochondrial electron transport. The mechanism of uncoupling action described lacks plant specificity and therefore works in all other types of oxygenic organisms. The inherent toxicity potential of these herbicides has consequently led to abandonment of the use of these compounds.

Uncoupling of oxidative phosphorylation has dramatic effects on seed germination and in other heterotrophic tissues, such as roots and buds. The breakdown of the energy charge in living cells leads to a breakdown of all life-maintaining systems and all energy-requiring anabolic reactions. Ribonucleic acid (RNA) and protein synthesis are rapidly inhibited. Nitrite accumulates in the tissue. The membrane electrochemical gradient and the semipermeability are lost, and the cell interior compounds leak into the surrounding tissue. The contents of cytoplasm, vacuole, and apoplast are mixed, and the vacuolar acids lower the prevailing pH, enabling hydrolyzing enzymes with acid pH optima such as lipases, proteases, esterases, and phosphatases to hydrolyze the cell components. This process is called *autolysis*.

### 3. Visible Symptoms: Signs of Decay

The visible symptoms can best be seen in germinating seeds: new growth is slow and the tissue then decays and becomes slimy. Since these seedlings have lost their metabolic power, the damaged tissue is often attacked by plant pathogens.

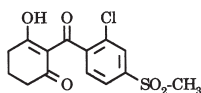
## III. INHIBITION OF CHLOROPLAST ISOPRENOID BIOSYNTHESIS

The herbicides shown in Fig. 14 induce white tissue in newly grown plant leaves in the light. Other herbicidal interactions not involved in the carotenoid biosynthesis pathway may also lead to different types of chlorosis, but these are not considered here. A selection of structures and herbicides inhibiting different steps of the carotenoid biosynthesis pathway in chloroplasts are shown. Only in the groups of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) and phytoene desaturase (PDS) inhibitors do we find a multitude of compounds and structures. Sulcotrione was known as a herbicide that causes chlorosis since 1985, before the primary target was eventually detected (45, 46). The elucidation of HPPD as a primary herbicide target was aided by the publication of a mammalian HPPD inhibitor (the triketone 2-[2-nitro-4-trifluoromethylbenzoyl]-cyclohexane-1,3-dione, NTBC) as a treatment for alcaptonuria (47), a disease caused by a defect in tyrosine metabolism (absence of the enzyme homogentisic acid oxidase). In addition, NTBC is a good herbicide. The story of HPPD herbicide discovery and development and the structure requirements for herbicidal diketones has been described (48–50). In the case of isoxaflutole, the herbicidally active structure is the opened ring form, the diketone nitrile (51, 52). A summary of carotenoid biosynthesis inhibitor herbicides has been published (53).

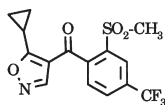
### A. Primary Targets

#### 1. Inhibition of 4-Hydroxyphenylpyruvate Dioxygenase

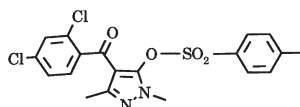
It may be surprising that the synthesis of homogentisic acid is the first target mentioned in this chapter on inhibition of isoprenoid biosynthesis. However, the pathway containing the enzyme HPPD eventually leads to the synthesis of plastoquinone (PQ/PQH<sub>2</sub>), which may be a necessary cofactor of the enzymes to be dealt with next, phytoene desaturase and  $\zeta$ -carotene desaturase (54). The mode of action of herbicides inhibiting HPPD therefore is in most aspects similar to that of herbicides inhibiting

**Inhibitors of 4-hydroxyphenylpyruvate dioxygenase (HPPD)**

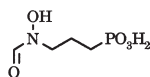
Sulcotrione



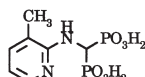
Isoxaflutole



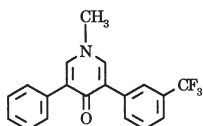
Pyrazolynate

**Inhibitors of DXP reductoisomerase and FPP synthase**

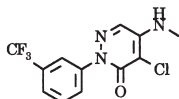
Fosmidomycin



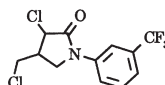
CGA 103586

**Inhibitors of phytoene desaturase (PDS)**

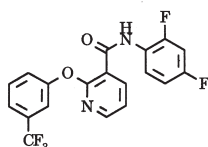
Fluridone



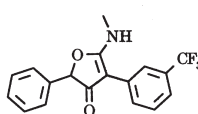
Norflurazon



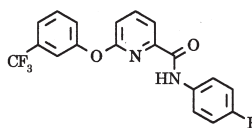
Flurochloridone



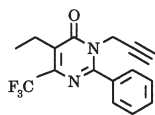
Diflufenican



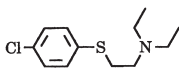
Flurtamone



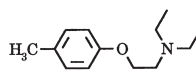
Picolinafen

**Inhibitors of ζ-carotene desaturase (ZDS) and lycopine cyclase (LCC)**

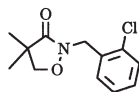
RH 1965



CPTA



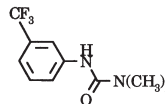
MPTA

**Unknown primary target**

Clomazone

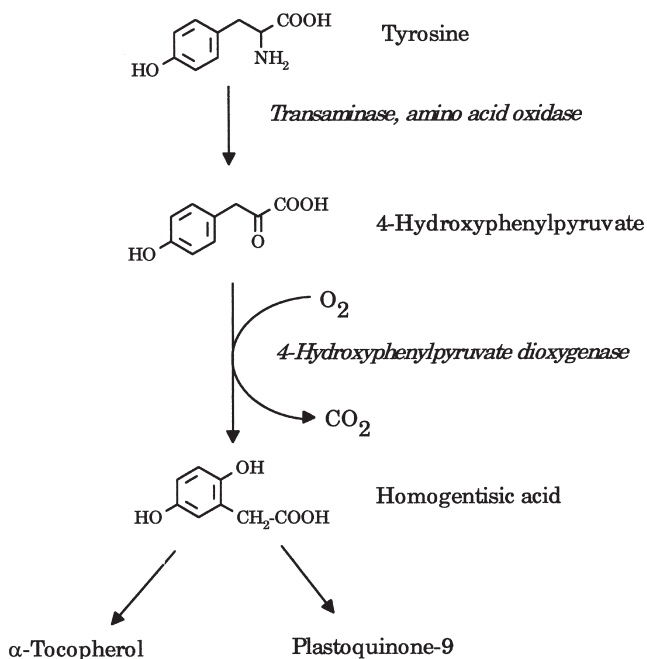


Amitrole



Fluometuron

**Figure 14** Structures of herbicides and some herbicidally active compounds that inhibit different enzymes in the biosynthetic pathway leading to the carotenoids. DXP, 1-deoxy-D-xylulose-5-phosphate; FPP, farnesylpyrophosphate.



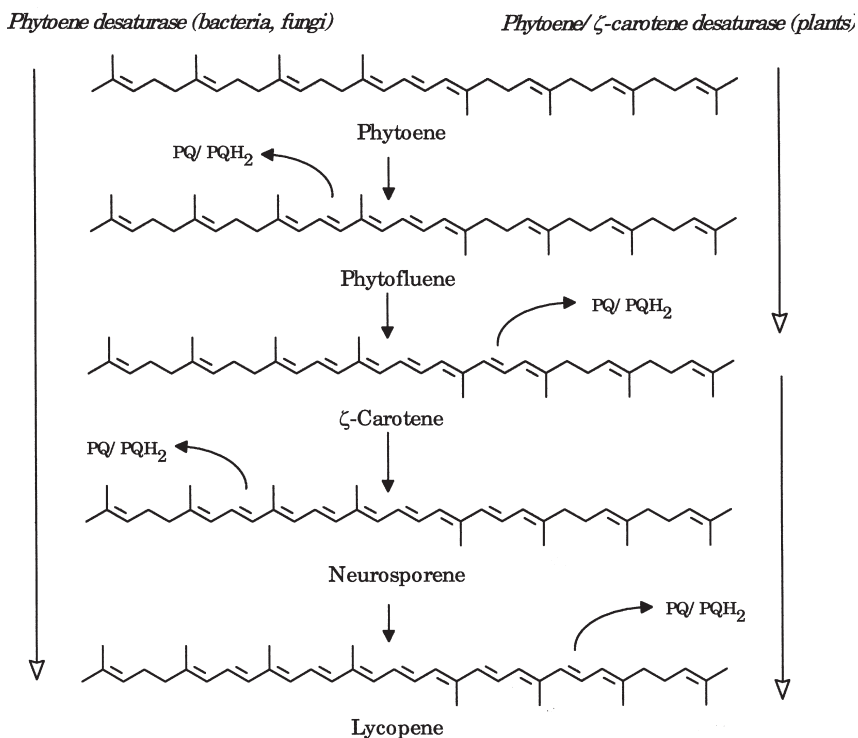
**Figure 15** Synthesis of homogentisic acid by 4-hydroxyphenylpyruvate dioxygenase (HPPD). The substrate 4-hydroxyphenylpyruvate is in turn a product of tyrosine catabolism, more specifically of transamination (plants) or amino acid oxidation (microorganisms).

these desaturases. However, HPPD inhibitor herbicides are growth inhibitors in addition to inducers of chlorosis, and the inhibition and chlorosis can be antagonized by homogentisic acid. Another aspect of the mode of action of HPPD inhibitors is the inhibition of  $\alpha$ -tocopherol synthesis (55).  $\alpha$ -Tocopherol is a scavenger of activated singlet oxygen and rapidly turns over in light. Its lack leads to D1 protein oxidation, oxidative tissue damage, and bleaching (see Sec. II.D).

The HPPD enzyme mechanism (Fig. 15) involves ring peroxidation, migration of the side chain into the *o*-position, and oxidative decarboxylation of the side chain (56). This is a special case of the dioxygenases, since the cosubstrate to be reduced by the second oxygen atom is in this case supplied by the side chain of the substrate. The inhibition is competitive with respect to the substrate and involves chelation of an enzyme-bound ferrous ion to form a stable ion-dipole charge transfer complex. The inhibition at the enzyme level occurs at nanomolar concentrations.

## 2. Inhibition of Phytoene Desaturase and $\zeta$ -Carotene Desaturase

The enzymes phytoene desaturase (PDS) and  $\zeta$ -carotene desaturase (ZDS) catalyze closely similar desaturations of their  $C_{40}$  prenyl lipid substrates (Fig. 16) (57). Even though two different enzymes exist in plants for the “inner” and “outer” desaturations, most of the herbicidal inhibitors probably inhibit both enzymes, although to a different extent. Strong inhibition of PDS leads to a block at the first substrate, phytoene, and its accumulation. The compound’s ability to inhibit ZDS can then not be observed *in vivo*. The compound RH 1965 has been reported to be a specific inhibitor of the second enzyme,  $\zeta$ -carotene desaturase (58). Other compounds claimed to inhibit ZDS have not been developed as herbicides (59).



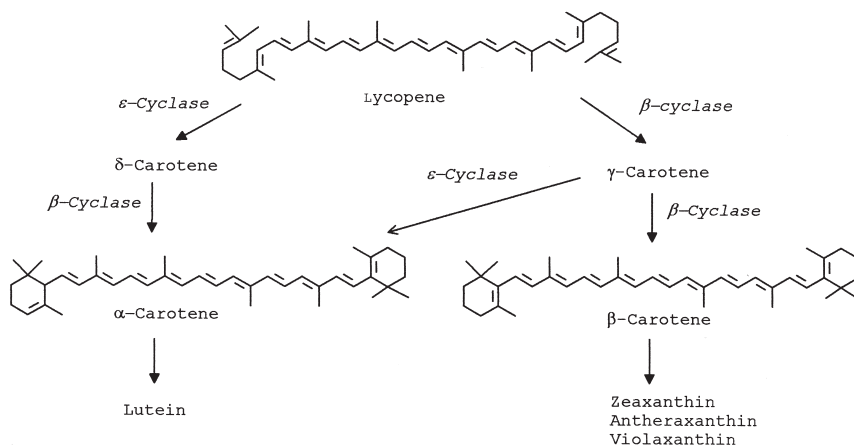
**Figure 16** Desaturation reactions in the carotenoid biosynthetic pathway from phytoene to lycopene (compare with Fig. 18). The first two “inner” and the second two “outer” desaturations are catalyzed by two different plant enzymes. PQ, oxidized plastoquinone; PQH<sub>2</sub>, reduced plastoquinone.

### 3. Inhibition of Lycopene Cyclase (LCC)

Cyclization of lycopene (Fig. 17) is accomplished by two different enzymes for the  $\beta$ - and the  $\epsilon$ -cyclization, called *lycopene  $\beta$ -cyclase* and  *$\epsilon$ -cyclase*, respectively ( $\beta$ - and  $\epsilon$ -LCC) (60, 61). The herbicides CPTA and MPTA appear to inhibit both enzymes. In 2001, new strong inhibitors of LCC were found (62). However, although very effective in *Chlamydomonas reinhardtii* and in seedling tests, the inhibitors of LCC known so far lack sufficient activity for herbicide development.

### 4. Clomazone: Primary Target Still Unknown

Clomazone is a bleaching herbicide for which the primary target is still not known, despite much research effort. Contrasting with the PDS inhibitors, clomazone inhibits growth of seedlings in the dark, in addition to inhibiting carotenoid biosynthesis. Since this inhibition can be antagonized by the addition of the diterpene derivative gibberellic acid, the herbicidal inhibition should occur before the synthesis of geranylgeranyl pyrophosphate (GGPP). The other diterpene derivative whose synthesis is also inhibited is the phytol side chain of chlorophyll. Examination of dark-grown seedlings under dim light reveals that treated seedlings are not completely white but are somewhat light green (63–65). The ultrastructure of chloroplasts is strongly



**Figure 17** Two different enzymes for  $\beta$ - and  $\epsilon$ -cyclization of lycopene. Although both are sensitive to inhibitors, the  $\beta$ -cyclase appears to be the more important for the herbicidal action, because of the importance of  $\beta$ -carotene as a chlorophyll protectant in strong light.

disturbed. Interestingly the synthesis of steroids, which are derived from the sesquiterpenoid farnesylpyrophosphate (FPP), is not affected (66, 67). Similarly, the synthesis of the sesquiterpenoid-derived phytoalexins gossypol and hemigossypol in cotton seedlings is not inhibited but is slightly enhanced by clomazone (68). No *in vitro* effects of clomazone have been found on any enzyme involved in terpenoid synthesis (69, 70). However, the results of these earlier experiments must be reinterpreted in the light of the finding that plastid and cytoplasm have been found to have different pathways to produce the building blocks of terpenoids, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (see Sec. III.B). If the target site is in the pathway before IPP and DMAPP, terpenoids produced by the cytoplasmic pathway are not affected, since the two pathways are the same after this point.

Lange and associates (71) found that clomazone completely inhibited formation of both IPP and DMAPP in peppermint oil gland secretory cells that have strong plastidic terpenoid synthesis of monoterpenes and sesquiterpenes. Unidentified intermediates accumulated. The enzymes of this part of the plastid pathway have not been tested for sensitivity to clomazone in an *in vitro* assay. If the results of such a study were negative, those results would suggest that clomazone is a proherbicide, with a metabolite inhibiting early in the chloroplast terpenoid pathway.

## 5. Amitrole: The Oldtimer

Amitrole is one of the early herbicides, developed and applied in the 1950s. In spite of this long history and much research, the primary site of interaction is still unknown. Amitrole is a nonselective herbicide that causes severe chlorosis. Phytoene, phytofluene,  $\zeta$ -carotene, and lycopene are all accumulated. Lycopene is only accumulated in minor quantities, much less than with the LCC inhibitors. As described for clomazone, the *in vitro* synthesis of lycopene is not inhibited. Also similarly to clomazone, steroid biosynthesis is not inhibited, but even the precursor squalene accumulates after treatment with amitrole. It may therefore also indirectly inhibit an early step in carotenoid biosynthesis. It also causes deregulation of the chlorophyll pathway, resulting in enhanced accumulation of protochlorophyllide in darkness (72), similar to the deregulation seen in dark-grown plants treated with PPO inhibitors (73). This effect is apparently due in each case to lack of feedback inhibition of the pathway by protoporphyrin. The PPO inhibitors block the pathway to protochlorophyll directly, whereas amitrole inhibits synthesis of the phytol component of protochlorophyll (74).

Amitrole irreversibly inactivates catalase at millimolar concentrations. At lower concentrations, a reversible inhibition occurs. This inhibition



certainly is not relevant to the herbicidal action but has been used in investigations of the plant metabolism (75, 76). Glutathione (GSH) is oxidized to GSSG, and hydrogen peroxide levels increase under photorespiratory conditions. The simultaneous accumulation of formate could arise from the oxidation of photorespiratory glyoxylate, leading to a carbon drain and inhibition of photosynthetic CO<sub>2</sub> fixation.

## 6. Fluometuron: The Bifunctional

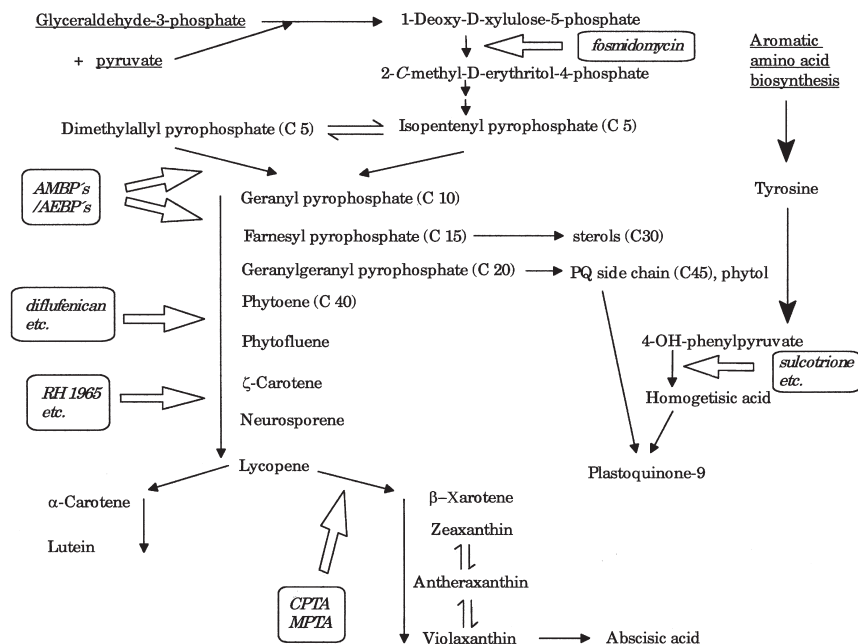
As a phenylurea compound, fluometuron is a strong inhibitor of photosynthetic electron flow in D1. The structure is shown in Fig. 4. However, fluometuron also causes carotenoid biosynthesis inhibition and bleaching in treated plants. The molecular mode of this inhibition has not been investigated.

## B. Affected Metabolism: The Complex Isoprenoid Network

In the late 1990s and 2001 it was found that the isoprenoid compounds in chloroplasts are synthesized from glyceraldehyde-3-phosphate and pyruvate by a condensation reaction leading to 1-deoxy-D-xylulose-5-phosphate (DXP) (77–79). The subsequent reaction is catalyzed by DXP reductoisomerase. On the contrary, cytoplasmic sterols are synthesized from cytoplasmic isopentenyl pyrophosphate, which is derived from the mevalonic acid route.

The isoprenoid polymerization sequence (Fig. 18) starts by head to tail condensation of the C<sub>5</sub> units IPP and DMPP to the monoterpene geranylpyrophosphate (GPP). The respective enzyme farnesylpyrophosphate synthase (FPPS) in most plants catalyzes two steps: the condensation to GPP and the next addition of one IPP to form the sesquiterpene farnesylpyrophosphate (FPP). The next step to the diterpene GGPP may in some plants be synthesized by a separate enzyme, geranylgeranylpyrophosphate synthase (GGPPS). The first carotenoid C<sub>40</sub> precursor compound phytoene is then synthesized by a head to head condensation of two GGPP, leading to the central double bond in phytoene.

In the described scheme, the starting compounds for the synthetic sequences are underlined. Both lines of synthesis convene in plastoquinone, which is probably required as a cofactor in the desaturations from phytoene to lycopene. A total of six different enzymes of carotenoid biosynthesis are so far known to be good herbicide target sites. They are highlighted in the scheme by open arrows and the names of representative inhibitory compounds. The DXP reductoisomerase is inhibited by the herbicidal natural compound fosmidomycin (80). The FPPS is inhibited by



**Figure 18** Biosynthetic pathways from the C 3 precursors glyceraldehyde-3-phosphate and pyruvate to the C 30, C 40, and C 45 end products, sterols, carotenoids, and phytol, respectively. Except the sterol biosynthesis branch, the pathways are located in the chloroplasts. Six different herbicidal inhibition points are shown by open arrows and typical herbicide names. AMBP, aminomethylene-bis-phosphonate; AEBP, aminoethylene-bis-phosphonate; PQ, plastoquinone.

experimental herbicides with aminomethylene- and aminoethylene-bis-phosphonate (AMBP, AEBP) structures (81, 82). These are, however, only weak herbicides because diphosphonic acids are not easily taken up by the plant. In this respect they are much more difficult to improve by formulation methods than glyphosate (compare Sec. IV.D). Additional bleaching compounds with unknown primary targets were discussed earlier.

### C. Physiological Effects: More Consequences of Excess Light

Chloroplasts are semiautonomous organelles that, besides photosynthetically fixing carbon, synthesize amino acids, fatty acids, chlorophylls, carotenoids, and other metabolites. Thus, it is not surprising to find the

targets for most of the well-known herbicides within the chloroplast and its membrane systems. Specifically, the carotenoid biosynthesis pathway proceeds in the chloroplast envelope membranes (83).

As can be expected, after treatment with herbicides that inhibit the carotenoid biosynthesis pathway, an accumulation of precursor compounds can be observed. The method of choice for analysis is high-performance liquid chromatography (HPLC) (84):

1. Phytoene accumulation occurs after inhibition of PDS, but also indirectly after inhibition of HPPD.
2.  $\zeta$ -Carotene and phytoene accumulate after treatment with an inhibitor of ZDS.
3. Lycopene and smaller amounts of phytoene and phytofluene accumulate after treatment with a LCC inhibitor.
4. The rapid turnover of the D1 protein is inhibited by the inhibitors of PDS, ZDS, and LCC.
5. Amitrole does not inhibit D1 turnover and leads to only very low levels of lycopene accumulation, as well as more phytoene and some phytofluene and  $\zeta$ -carotene; the primary target of amitrole is not known.
6. Simultaneously, chlorophylls are broken down oxidatively in the light because their protective carotenoids have been lost; as described in some detail in Sec. II.D on oxygenic damage after overloading and/or loss of repair systems, carotenoids have a protective function for chlorophylls.
7. It makes a big difference whether plants are treated before or after emergence, and whether in light or darkness: (a) in the dark, the etiolated tissue shows the accumulations described in 1–3; (b) in the light, chlorosis is observed, but no such accumulations can be measured; apparently, oxidation products created early during illumination of treated tissue in the proplastids downregulate the further chloroplast development (85).
8. When dark- or light-grown plants are treated later (after emergence), little herbicidal action can be seen because carotenoid biosynthesis has already occurred; however, new growth shows the symptoms described.
9. Treatment with fosmidomycin reduces all isoprenoid synthesis, including, for example, gibberellin biosynthesis, and has additional metabolic and visual effects.
10. Similarly, treatment with HPPD inhibitors has specific effects: (a) homogentisic acid can alleviate the inhibition by the herbicide; (b) the contents of plastoquinone and  $\alpha$ -tocopherol both decrease

in emerging leaves; (c) eventually, phytoene accumulates in the treated tissue and chlorosis occurs (51).

Some inhibitors of PDS are also known to inhibit other desaturases, specifically fatty acid desaturases, for the step from linolenic to linoleic acid (compare Sec. VI.A and Fig. 34). This desaturation inhibition alters low-temperature acclimation, because of a decreased membrane fluidity (86, 87). Of particular interest are norflurazon analogs with a low specificity for PDS but a high specificity for fatty acid desaturases (FDS).

#### D. Visible Symptoms: “Real” Bleaching

Herbicides that inhibit carotenoid biosynthesis are also called *bleaching herbicides* because of the white tissue that develops after treatment. However, bleaching can occur after inhibition of many different primary targets. For example, glyphosate treatment leads to bleached leaves under certain conditions, and PS II and PPO inhibitors bleach leaves when the herbicidal action develops slowly. However, highly active and specific PDS inhibiting herbicides lead to completely white tissue that is not damaged otherwise. Only 1–3 days later the tissue begins to wither because no photosynthesis occurs. Herbicides with other primary targets in carotenoid biosynthesis, either directly or indirectly, have additional effects such as dwarfing or light green to yellow-white coloring, turning white to brownish and necrotic with time.

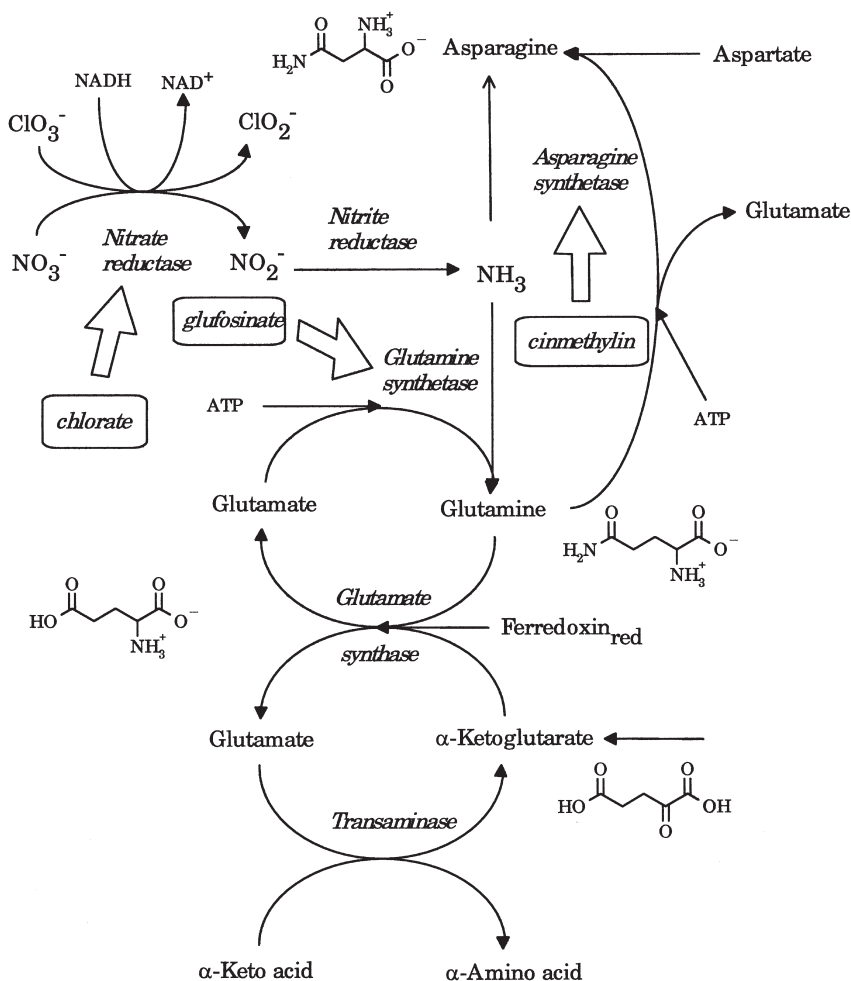
After PDS inhibition in weak light, chloroplasts are developed and the tissue turns green because the light requirement of chlorophyll biosynthesis is satisfied, and protection of chlorophylls by carotenoids is not required. When this tissue is subjected to strong light,  $^1\text{O}_2$  is produced by energy transfer from excited chlorophyll triplet stages and peroxidation of cell constituents takes place: chlorophylls, endomembranes, and 70S chloroplast ribosomes are rapidly destroyed, and the tissue decays and turns necrotic and brown.

### IV. BIOSYNTHESIS OF AMINO ACIDS

#### A. Targets in the Metabolic Supply of Reduced Nitrogen

##### 1. Primary Targets, Affected Metabolism, and Physiological Effects: Differences in Secondary Effects

The introduction of nitrogen into the amino acid and other plant metabolism (Fig. 19) usually starts with reduction of nitrate by nitrate reductase (88). Nitrate reductase is a highly regulated enzyme, which is only



**Figure 19** Nitrate reduction and introduction of reduced nitrogen into the nitrogen metabolism of plants. Glutamine serves as a central metabolite in the route supplying amino groups for the synthesis of new amino acids from their respective  $\alpha$ -keto acids. Asparagine, however, seems to be more important as an amino group pool for long-distance transport and for depot functions. Enzyme and herbicide names are in *italic*. The three herbicidal targets are indicated by open arrows with the names of the herbicidal inhibitors.

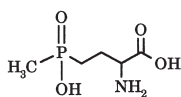
active in tissue with active nitrite reduction, to prevent toxic nitrite accumulation, especially in green leaves, where nitrite reduction is dependent on the photosynthetic electron flow.

Herbicide target enzymes are marked in the scheme by open arrows. Sodium chlorate ( $\text{NaClO}_3$ ) is a nonselective herbicide acting through nitrate reductase (NR): it is reduced to the highly phytotoxic chlorite ion ( $\text{ClO}_2^-$ ), which rapidly destroys nitrate reductase and other enzymes and proteins nearby. However, fairly high doses (up to  $20 \text{ kg ha}^{-1}$ ) are required as a result of its low specificity. Chlorate ( $K_M$   $1.2 \text{ mM}$ ) is a competitive analog to nitrate, not only at the enzyme level, but also during its uptake from the soil. Resistance observed after selection in the presence of chlorate has been shown to follow three mechanisms: (a) uptake defects with a reduced activity of the uptake carrier in *Arabidopsis thaliana* (89), (b) resistant NR enzymes (90, 91), and (c) NR activity regulation defects (92).

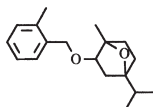
Ammonia is supplied to the tissue (a) by the nitrate reduction enzymes, (b) after catabolic amino acid breakdown, and (c) during photorespiratory recycling of aspartate or glutamate. The assimilation of ammonia is carried by the coordinate activity of glutamine synthetase and glutamate synthase, also called *ferredoxin-dependent glutamate oxoglutarate aminotransferase* (Fd-GOGAT) (93, 94). From there, amino groups are transferred to other keto acids during the synthesis of further amino acids, and to other acceptors for the amino group.

Glutamine synthetase (GS) is the target of the nonselective herbicide glufosinate (Fig. 20). Phosphinothricin is the active L-isomer of glufosinate, whereas bialaphos is a naturally occurring tripeptide precursor containing two alanines (95). The herbicides are structural analogs of glutamate, inhibiting GS at  $5\text{--}10 \mu\text{M}$ . The inhibition of GS leads to the accumulation of toxic amounts of ammonia in leaf tissue, especially during active photorespiration. High ammonia concentrations also uncouple photophosphorylation. Photosynthetic  $\text{CO}_2$  fixation is rapidly inhibited, and amino acid pools are affected strongly. Photorespiration suffers from a lack of the

#### Inhibitors of glutamine synthetase and asparagine synthetase



Glufosinate



Cinmethylin

**Figure 20** Structures of two herbicides that inhibit the biosynthesis of amino acid amides. The amino acid amides glutamine and asparagine both serve to supply high-energy amino groups for biosynthetic routes and/or for long-distance transport.

amino acids alanine, serine, aspartate/asparagine, and glutamate/glutamine (96, 97). Summing up, the inhibition of photosynthesis and photorespiration appear to be the result of effects on the carbon cycling in these pathways. Ammonia accumulation has also been used as an index in the selection of glufosinate-resistant cell lines (98).

The third herbicidal target enzyme in the primary assimilation of nitrate and ammonia is asparagine synthetase (AS), which is inhibited by a metabolite of cinmethylin (Fig. 20) and by other cineoles (99, 100). Asparagine is an important transport form of reduced nitrogen in plants and during reserve mobilization in germinating seeds. The actual enzyme inhibitor is the hydrolysis product *cis*-2-hydroxy-1,4-cineole. The parent compound cinmethylin is not inhibitory to the enzyme and therefore is a proherbicide. Also, only the benzyl ether apparently has the lower volatility and stability that are required for a commercial herbicide. Of the two plant enzymes, one is specific for glutamine, whereas the second can use either glutamine or ammonia. The inhibitor appears to bind to the glutamine binding site, since, besides asparagine, glutamine antagonizes the herbicidal action to some extent. The metabolism of cinmethylin is very complex in that many metabolites, of which many have not been identified, are generated within the first minutes to hours. The positive correlation between metabolite accumulation in the tissue and herbicidal action has therefore yet to be shown.

## 2. Visible Symptoms: From Phytotoxicity to Meristem Sensitivity

The herbicidal effects exerted by the three different mechanisms described differ considerably:

1. After chlorate reduction by nitrate reductase, as mentioned, chlorite rapidly destroys the cell contents and the tissue. Rapid desiccation of leaf tissue in the light is observed. The concentrations required are rather high, in the 0.1- to 1-mM range, and the amounts to be applied in the field were therefore in the 5- to 10-kg range.
2. Since chloroplasts with active photorespiration have a high ammonia turnover in the light, inhibition of glutamine synthetase leads to a buildup of high concentrations of free ammonia and ammonium ions in the leaves, up to  $100\ \mu\text{mol g}^{-1}$  fresh weight. Membranes particularly in chloroplasts are depolarized, lose their semipermeability, and are rapidly destroyed. After a few hours, yellowing, chlorosis, and necrosis occur.
3. Cinmethylin inhibits the onset of mitosis and cell division. A decrease of all mitotic stages is observed. Cell enlargement is not specifically inhibited. Roots are ca. 10 times more sensitive than

shoots. These observations should eventually be traced to the inhibition of asparagine synthetase, but because of the very recent uncovering of this primary target, no such work has so far been published. The functions of asparagine are in long-distance transport and in the availability of reduced nitrogen in developing seedlings and in leaf and shoot tissue. Roots, which are especially dependent on this supply, may therefore suffer more than shoots. Meristematic tissue is probably most sensitive because of the high rates of amino acid metabolism and protein synthesis.

## B. Inhibition of Acetolactate Synthase

When first detected in 1977 at Dupont, sulfonylureas were examples for a new generation of herbicides with very low application rates ( $5\text{--}50\text{ g ha}^{-1}$ ) and low mammalian toxicity. As we now know, this is realized because of (a) the high affinity to the target enzyme and (b) the low amounts of target enzyme present in sensitive tissues.

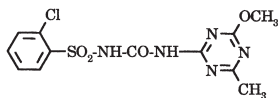
The structures of herbicides that inhibit acetolactate synthase are very diverse (Fig. 21). Two ways to categorize this diversity are either (a) into groups with or without a necessary free carboxy group or (b) into five commonly divided structure groups, as done here. In cases in which the carboxy groups of imidazolinones and pyrimidyl(thio)benzoates are esterified, these are then inactive proherbicides. However, when dissolved in water, herbicides from the three other structural groups also behave as acids since the proton in the structural element  $\text{--SO}_2\text{--NH--CO--}$  or a similar element readily dissociates. However, esterified carboxy groups in the latter structures, as in bensulfuron-methyl, are required for activity. Free acids in this position mean loss of inhibitory and herbicidal activity. More examples can be seen at the HRAC Internet site (2). A survey in 2000 applied 3D methods to create a model for the three-dimensional characteristics of the different ALS inhibitor groups (101). The total number of individual structures that could inhibit ALS and be active as herbicide appears to be nearly unlimited; many thousands have been tested. From this enormous number, some of those with good selectivity in major crop plants were developed into commercial products.

### 1. Primary Target: Entering the Biosynthetic Pathway

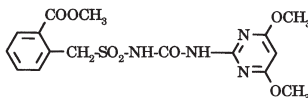
The enzyme inhibited by the herbicides of this group starts the biosynthesis pathway leading to the branched chain amino acids. It is a condensation



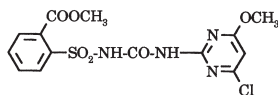
## Sulfonylureas



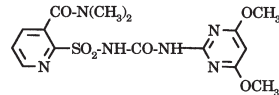
Chlorsulfuron



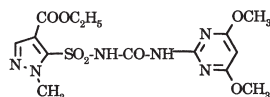
Bensulfuron-methyl



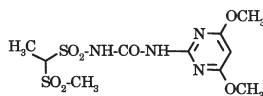
Chlorimuron-methyl



Nicosulfuron

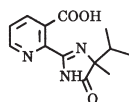


Pyrazosulfuron-methyl

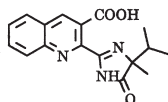


Amidosulfuron

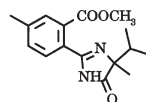
## Imidazolinones



Imazapyr

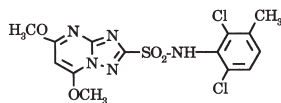


Imazaquin

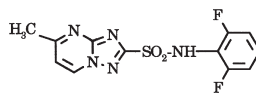


Imazamethabenz-methyl

## Triazolopyrimidines



Metosulam

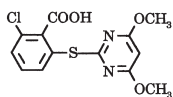


Flumetsulam

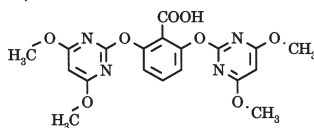
**Figure 21** Examples of herbicidal inhibitors of acetolactate synthase (ALS) from five different structure groups. These 15 structures are only a small sample of the ca. 60 known commercial ALS inhibitor herbicides. The biggest group (ca. 40) is formed by the sulfonylurea compounds.

reaction, as described in Fig. 22. Two separate pathways lead to either valine and leucine or to isoleucine, but the same enzyme catalyzes the first reaction to either 2-acetolactate or 2-acetohydroxybutyrate. Thus, the enzyme has obtained two names: *acetolactate synthase* (ALS) and *acetohydroxyacid synthase* (AHAS) as a general name.

## Pyrimidyl(thio)benzoates

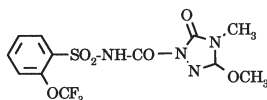


Pyriithiobac

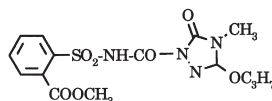


Bispyribac

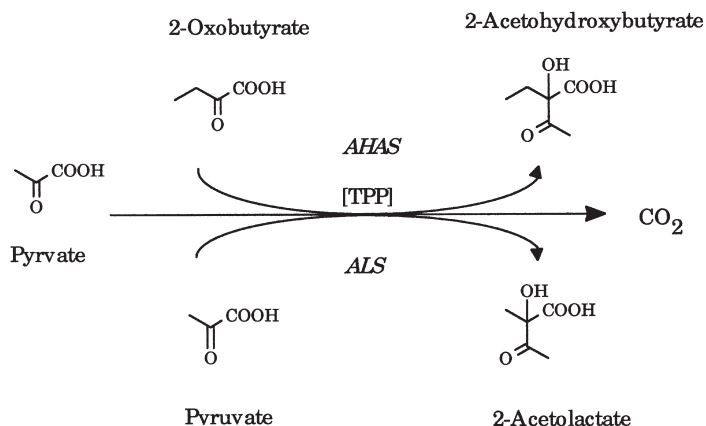
## Sulfonylaminocarbonyl-triazolinones



Flucarbazone



Propoxycarbazone

**Figure 21** Continued.

**Figure 22** The enzyme acetolactate synthase (ALS) catalyzes the starting reactions for the synthesis of all branched chain amino acids, valine, leucine, and isoleucine (compare with Fig. 23). It therefore catalyzes both reactions, either to valine and leucine via 2-acetolactate (ALS reaction proper) or to isoleucine via 2-acetohydroxybutyrate. The name *acetohydroxyacid synthase*, AHAS, is occasionally also used to include both reactions. Thiamine pyrophosphate (TPP) is a cofactor of the reaction that is required for the decarboxylation.

Acetolactate synthase is a thiaminepyrophosphate (TPP) enzyme with a TPP-hydroxyethyl intermediate after  $\text{CO}_2$  elimination; it is usually tested by the measurement of acetolactate and therefore abbreviated *ALS*. The ALS inhibitor herbicides usually inhibit increasingly with time and at nanomolar concentrations ( $k_i$  5–20 nM). However, imidazolinone structures

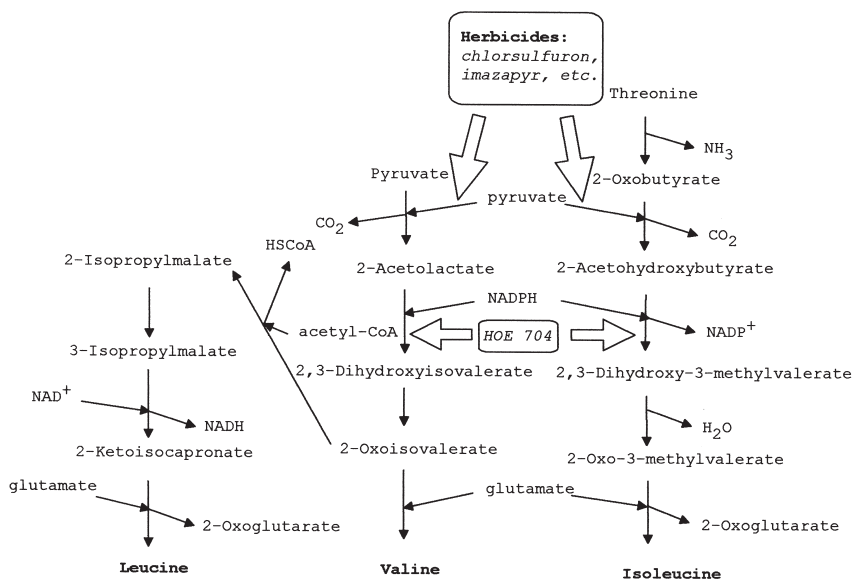
are much less active at the isolated enzyme level ( $k_i$  1–10  $\mu\text{M}$ ). Still, in vivo imidazolinones inactivate the enzyme after a few hours at nanomolar concentrations (102). During enzyme catalysis, the herbicides are thought to inhibit the condensation of the TPP-bound decarboxylation product of the first pyruvate (activated acetaldehyde) with either the second pyruvate or 2-oxobutyrate. The inhibition is not competitive. Schloss has suggested that the binding of the ALS herbicides involves a quinone binding site or niche close to the enzyme-bound flavin adenine dinucleotide (FAD), which is a “fossil” inherited from the enzyme pyruvate oxidase when it evolved into ALS (103). This arrangement could explain the vast variety of structures inhibiting ALS, largely similar to the D1 protein in photosystem II, which also contains a quinone binding niche adapted by many diverse herbicide structures (see Sec. II.B).

## 2. Affected Metabolism: Branched Chain Amino Acid Biosynthesis

The synthesis of branched chain amino acids derives from pyruvate and in the case of isoleucine also from threonine. The latter pathway forms part of the aspartate group amino acids (104).

A total of eight different enzymes catalyze the conversion of the precursors threonine and pyruvate into the branched chain amino acids valine, leucine, and isoleucine (Fig. 23), with the additional input of redox catalysts (NADPH and oxidized nicotinamide adenine dinucleotide [ $\text{NAD}^+$ ]), acetyl coenzyme A (acetyl-CoA), and the amino groups of glutamate. The enzyme ALS/AHAS and the next two enzymes serve both pathways, to valine/leucine and to isoleucine.

As is the case for many other herbicide primary targets, ALS inhibitors can also lead to the selection of resistant weeds. This effect occurs mainly with herbicides that do not degrade rapidly in the environment and exert their selection pressure over many months or years. In the case of the ALS inhibitors, this process has occurred mainly with chlorsulfuron. The HRAC Internet site (2) gives an up-to-date overview of the situation in different countries of the world. The number of resistant weed species is presently about 50. The resistance mode in field situations was always a mutation leading to amino acid changes and rendering the enzyme resistant (loss of herbicide binding). Resistance factors commonly range between 10 and 100 but may be up to 1000. The resistance factors are different for different structural groups: resistance selection by chlorsulfuron leads to enzymes with unchanged or even increased sensitivity to imidazolinones and pyrimidyl(thio)benzoates (105). This relationship is similar to the situation of D1 mutants in photosystem II, which also show comparable differential



**Figure 23** This scheme gives a condensed overview of the pathways leading to the branched chain amino acids. Compounds in the main stream from the precursors pyruvate and 2-oxobutyrate to the amino acids are written from top to bottom; cofactors and second substrates are written on the sides. The herbicide target enzyme acetolactate synthase together with two typical herbicides is shown on top by an open arrow. The second herbicide target enzyme, acetohydroxyacid reductoisomerase (AHRI, Sec. IV.C), is similarly shown.

resistance against different structure groups (see Sec. II.B). Many cell cultures were artificially selected with ALS-inhibiting herbicides, also leading to a variety of different resistant enzymes with very different resistance patterns.

### 3. Physiological Effects: Many Diverse Implications

The following consequences of ALS inhibition by herbicides have been found for metabolism and for cell growth and cell division:

1. An accumulation of 2-oxobutyrate and of 2-aminobutyrate as its transamination product: Genomic results in 2000 suggest that accumulation of these intermediates causes at least part of the toxic effect of ALS inhibitors (106).
2. An often only temporary reduction in the levels of the free amino acids valine, leucine, and isoleucine (particularly in meristems),

followed by a dramatic change of other free amino acid pools because of regulatory feedback effects.

3. Reversal of inhibition by valine and isoleucine in meristematic tissue only: the complexity of the regulation of amino acid metabolism and other dependent anabolic metabolism may be the reason for the frequent failure to antagonize growth inhibition with valine and isoleucine. Also, protein degradation and branched chain amino acid recycling occur in some tissues.
4. Inhibition of deoxyribonucleic acid (DNA) synthesis by ca. 80% after 5–7 h: since this inhibition occurs very rapidly and strongly, it may reflect branched chain amino acid starvation and consequences therefrom in certain subcellular pools only.
5. Decrease of the mitotic index and arrest of cells in the  $G_1$  and  $G_2$  stages, i.e., inhibition of the cell cycle stages  $G_1 \rightarrow S$  and  $G_2 \rightarrow M$  (G, growth; S, DNA synthesis; M, mitosis).
6. Inhibition of RNA synthesis by ca. 20%–40%, but no effect on protein and lipid synthesis rates.
7. Increase of protein turnover and levels of soluble amino acids by up to 70%.
8. Decrease of soluble protein levels by up to 40%.
9. Increase of free reducing soluble sugar and sucrose levels by 40%–50%.
10. Strong inhibition of phloem loading and phloem transport.
11. Inhibition of respiration by up to 30%.

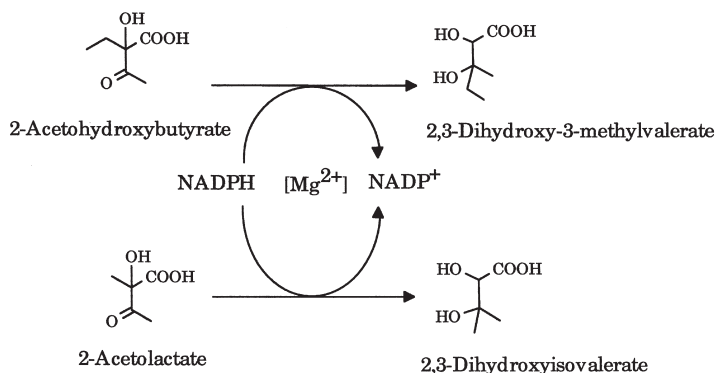
#### 4. Visible Symptoms: Growth Inhibition and Decay

Plants treated with inhibitors of ALS stop growth after a few hours. Tissue and leaves lose turgor and then appear droopy. After several days, field-treated seedlings often turn red as a result of the accumulation of stress anthocyanins. The symptoms are otherwise not very specific and reflect a general loss of growth rate and vitality.

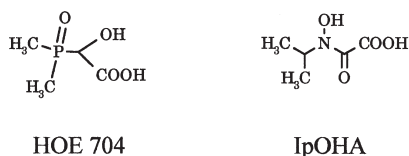
### C. Inhibition of Acetohydroxyacid Reductoisomerase

#### 1. Primary Target: Branched Chain Amino Acid Biosynthesis Step 2

The enzyme reaction in branched chain amino acid biosynthesis immediately after ALS/AHAS (Sec. IV.B) is catalyzed by acetohydroxyacid reductoisomerase (AHRI). This reaction is presented in Fig. 24 in its two pathways leading to either valine and leucine (2-acetolactate) or isoleucine



**Figure 24** The enzyme reaction catalyzed by acetohydroxyacid reductoisomerase (AHRI) is the second step in the pathway leading to the branched chain amino acids (compare Fig. 23).



**Figure 25** Structures of an experimental herbicide and an enzyme inhibitor for the target enzyme acetohydroxyacid reductoisomerase.

(2-acetohydroxybutyrate). The reaction involves migration of the alkyl side chain. It requires magnesium and NADPH for the reduction of the keto group. The  $\text{Mg}^{2+}$  coordinates the adjacent oxo/hydroxy groups when the alkyl side chain is transported into the adjacent position (107). The same is true for the binding of and inhibition by HOE 704 and *N*-isopropyl oxalylhydroxamate (IpOHA) (Fig. 25). HOE 704 inhibits the enzyme by 50% at 10–20  $\mu\text{M}$ ; inhibition is three orders of magnitude weaker than the inhibition of ALS by the respective herbicides.

## 2. Affected Metabolism, Physiological Effects, and Visible Symptoms: Searching for the Chain of Effects

The nonselective experimental herbicidal compound HOE 704 inhibits branched chain amino acid biosynthesis one step after ALS at AHRI (Fig. 23). HOE 704 leads to the accumulation of massive amounts of the substrate acetolactate and its decarboxylation product, acetoin (108).

However, the effects seen in treated plants are quite different from those of the ALS inhibitors. The herbicidal action develops very slowly and gradually leads to growth cessation, but without clear phytotoxicity. Since plants have grown to a medium size during this time, HOE 704 was suggested for use as a growth regulator. It is presently unknown for AHRI- as well as ALS-inhibiting herbicides how the herbicidal and visual symptoms are connected metabolically to the primary inhibition.

#### D. Inhibition of 5-Enolpyruvylshikimate-3-Phosphate Synthase

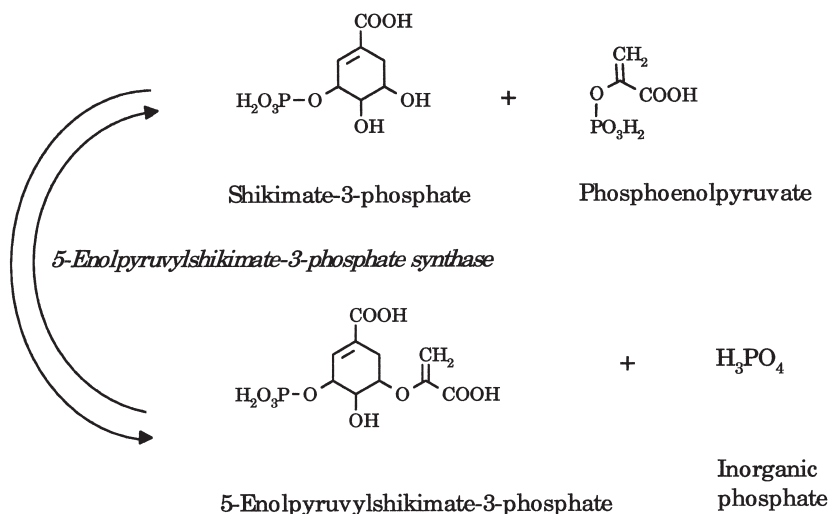
Glyphosate (Fig. 26) is the most successful herbicide of all times (109, 110). It was discovered in 1971 at Monsanto Company and was developed as a nonselective postemergence herbicide in orchards, vineyards, and other (semi)total herbicide uses and in minimum tillage agriculture. A new and successful use is application in genetically engineered herbicide-resistant crops (111). Glyphosate has little or no soil activity and disappears rapidly from the environment. It is a low-toxicity, low-cost herbicide with a largely plant-specific target in aromatic amino acid biosynthesis, the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Since this herbicide is easily translocated in the phloem and accumulates in subterranean regenerative organs such as rhizomes or tubers, it also kills perennial weeds such as *Rumex*, *Elymus*, and *Cyperus* species and prevents their regrowth. Glyphosate exists as an amino acid zwitterion at physiological pH, and many formulations as salts or esters have been developed to overcome the problems of its uptake from the leaf surface. Since it is also a divalent anion, it complexes strongly with divalent metal cations. It is normally sold as the isopropylamine salt. The trimethylsulfonium salt sulfosate is one of the possible formulations with improved uptake.



**Figure 26** Herbicidal inhibitors of aromatic amino acid biosynthesis at the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) step. Only the glyphosate structure is known to have herbicidal action; sulfosate is the trimethylsulfonium salt of glyphosate. Other specific EPSPS enzyme inhibitors found in subsequent synthesis programs did not have herbicidal activity.

### 1. Primary Target: An Enzyme Required for the Mass Flow of Carbon into Plant Aromatics

The primary herbicide target of glyphosate was found by Amrhein and associates (112–114). The target enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Fig. 27) is an enzyme in the shikimic acid pathway leading to the aromatic amino acids phenylalanine, tyrosine, and tryptophan and to phenylpropanoid metabolism (115). It is a chloroplast enzyme that has to be imported from the cytoplasm as a precursor. First EPSPS reacts with shikimate-3-phosphate and then with phosphoenolpyruvate (PEP). The enzyme reaction is reversible. Glyphosate binding is competitive with PEP in the forward reaction. In the reverse reaction, glyphosate is competitive with inorganic phosphate. Glyphosate is not a general PEP analog because it only inhibits EPSPS but no other PEP enzymes, and because PEP and glyphosate binding are apparently not identical. Glyphosate is probably bound to the enzyme site for binding of inorganic phosphate. The EPSPS enzymes from different organisms differ strongly in their glyphosate sensitivity, with  $k_i$  between 80 nM in *Pisum sativum* and 1–5  $\mu$ M in microorganisms. The  $k_m$  values for PEP do not show this variation, supporting the conclusion that glyphosate binding is not competitive with PEP. This has been called an *adventitious allosteric interaction*.



**Figure 27** The reversible enzyme reaction inhibited by glyphosate is a condensation in the forward and a phospholysis in the reverse reaction. It is an important step in the biosynthesis of aromatic amino acids (Fig. 28).



In spite of tremendous efforts, no other herbicides that inhibit EPSPS have been found. However, very effective transition state inhibitors have been found for this enzyme with nanomolar inhibition constants. These are inactive as herbicides in intact plants.

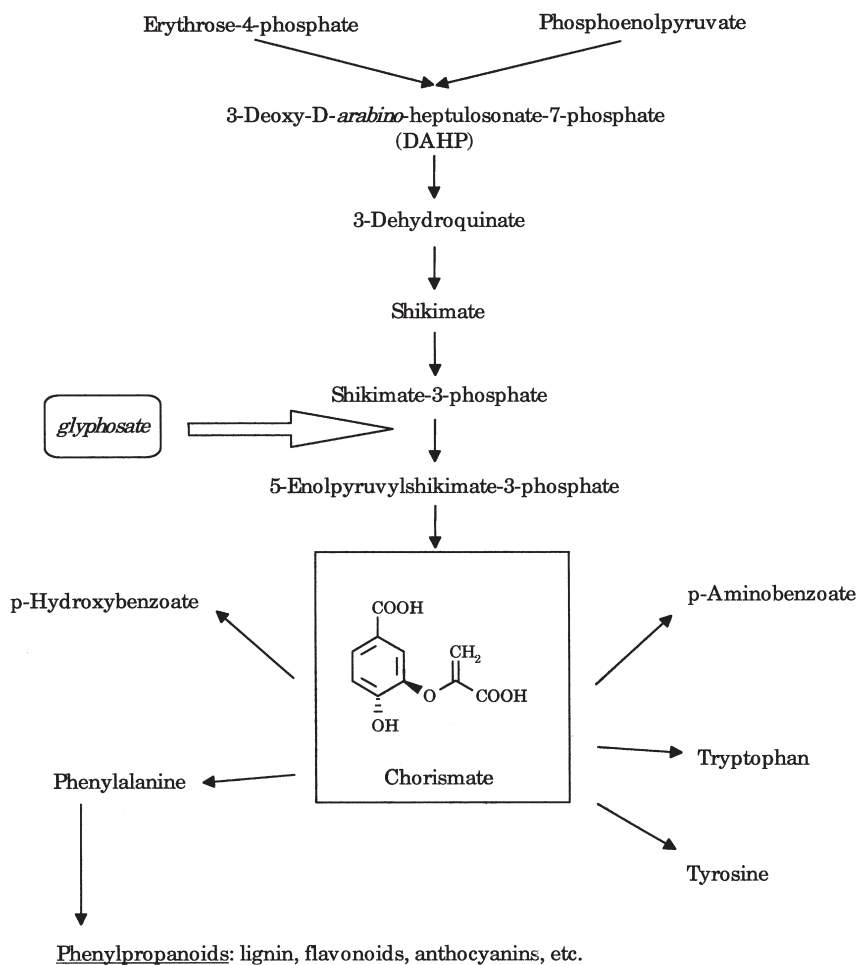
## 2. Affected Metabolism and Physiological Effects:

### Phenylpropanoid Metabolism for Flower Colors, Lignin, and Other Secondary Metabolites

The shikimate pathway not only supplies the growing plant with the aromatic protein amino acids, but also produces vast amounts of secondary metabolites with aromatic ring structures (Fig. 28) (116, 117). Among these are lignin, alkaloids, phytoalexins, ultraviolet (UV) light protectants, and anthocyanins. These secondary metabolism pathways all start with the enzyme phenylalanine ammonia lyase (PAL), which converts phenylalanine to cinnamic acid. Because PAL is an important regulatory enzyme it has been studied intensely in glyphosate-treated tissue. The activity of PAL is increased in glyphosate-treated tissues that suffer from low levels of phenylalanine. Since, however, inhibitors of PAL, such as L- $\alpha$ -aminooxy- $\beta$ -phenyl propionic acid (AOPP), also lead to elevated PAL levels, the enzyme may actually be controlled by the levels of cinnamic acid and/or its metabolites (118, 119). The very complex regulation of the described secondary metabolism is certainly the reason for the failure in many experiments to find the described secondary effects of glyphosate on plant metabolism. The situation is further complicated by the existence of several different PAL isoenzymes, which are separately regulated in different types of tissue.

An immediate consequence of the herbicidal action of glyphosate is the accumulation of the precursor molecule shikimate (up to 16% of the dry weight in sink tissues in which glyphosate accumulates). The shikimate accumulation obviously indicates that the immediate precursor shikimate-3-phosphate is dephosphorylated by an unspecific phosphatase. As a result of the complex regulation of amino acid and phenylpropanoid metabolism, and of the possible protein degradation and recycling of amino acids, the aromatic amino acids may be decreased or not decreased, depending on the tissue. A decrease of these amino acids is most easily found in rapidly growing meristematic tissue, such as in roots of maize or soybean or in cell cultures.

The dephosphorylation of shikimate-3-phosphate and accumulation of shikimate in very large amounts represent a considerable energy drain for the herbicide-treated plants. Also, the uncontrolled carbon flow into the shikimate pathway may negatively affect other important anabolic metabolic pathways in growing tissue. Since the addition of phenylalanine



**Figure 28** This scheme can give only a glimpse of the importance of phenylpropanoid metabolism for plant growth, structure, and defense. The message is that the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (open arrow) and of phenylalanine synthesis by glyphosate affects not only protein synthesis, but much more strongly the many other outlets for aromatic ring structures in plants.

and other aromatic amino acids to glyphosate-treated tissue does not always clearly antagonize the herbicidal effect, the herbicidal action may be a consequence of the deregulation of metabolism. As mentioned, the complex regulation of aromatic and phenylpropanoid metabolism is tuned to the

plant's needs in different tissues and situations. The lack of intermediates in the arogenate pathway from chorismate to phenylalanine and tyrosine in chloroplasts may also trigger and/or negatively affect regulatory switches (120). The massive accumulation of shikimate, along with rapid depletion of photosynthetic carbon reduction cycle intermediates (121), suggests that shikimate is a sink for misdirected carbon flux. Exogenously supplied arogenate substantially reduces glyphosate-induced shikimate accumulation in some tissues (120). The integration of chloroplast and cytoplasmic metabolism, which is instrumental in biosynthesis of many critical metabolites, is almost certainly lost. In conclusion, the herbicidal mechanism of glyphosate probably is the consequence of a massive deregulation of the plant's biosynthetic pathways, leading to the heavily branched and very important phenylpropanoid metabolism.

### 3. Visible Symptoms: Very Slow Tissue Degradation

The most rapid effect of glyphosate in rapidly growing young leaf tissue is a swelling of chloroplasts after ca. 20 h (122). Electron microscopic investigations also showed that chloroplast structure was greatly disrupted after 4 days. The growth of the young leaves and plants stops after some hours to days. Yellowing of the leaf and measurable chlorophyll breakdown start several days later.

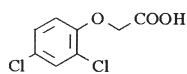
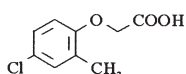
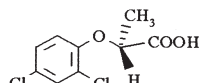
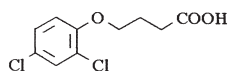
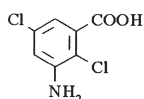
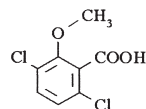
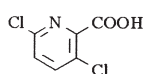
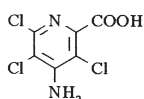
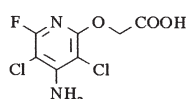
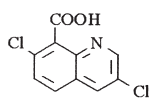
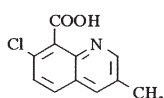
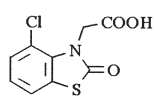
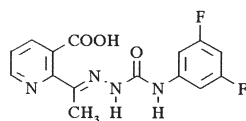
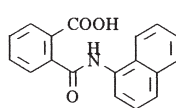
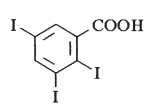
## V. AUXIN HERBICIDES

All auxin herbicides contain a free carboxylic acid group (Fig. 29). Sometimes the acid may be masked by esterification or amidation. For example, benazolin is applied as the ethyl ester. In other cases such as that of 2,4-DB,  $\beta$ -oxidation is necessary to shorten the butyric acid side chain to the corresponding acetic acid (2,4-D). Dichlorprop-P is the herbicidally active (+)-D-enantiomer of racemic dichlorprop. Whereas auxin herbicides are mimics of the natural auxin growth hormone indole-3-acetic acid (IAA), the auxin transport inhibitors must obviously differ from the "aspartate group amino acid" auxins, possibly by a more selective binding to certain receptors only.

The auxin transport inhibitors inhibit auxin efflux from the tissue by binding to an auxin efflux receptor (123, 124).

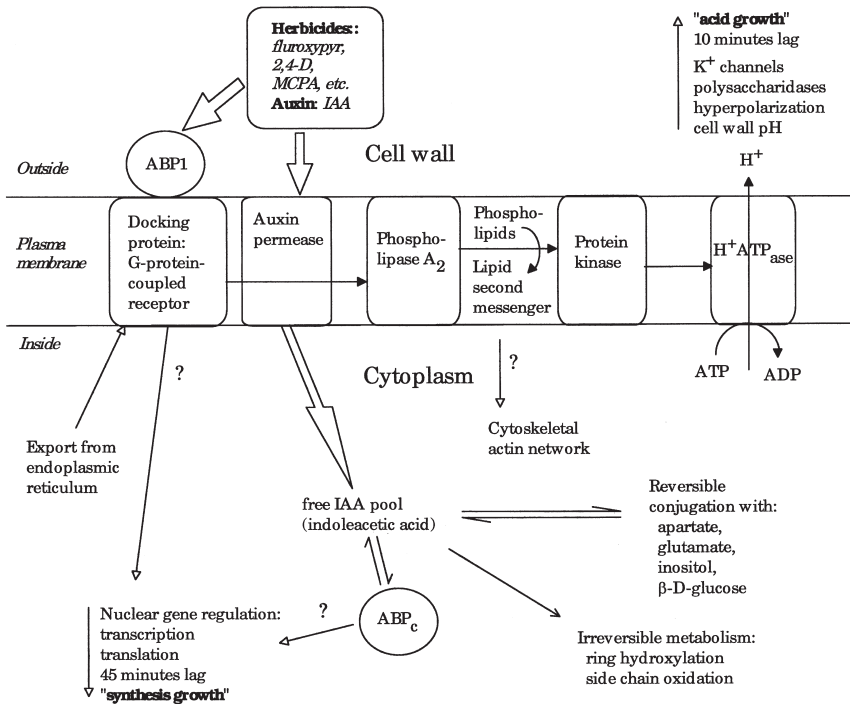
### A. Primary Target: How Many Auxin Binding Proteins?

The free auxin pool in plant tissue is highly regulated by synthesis as well as reversible and irreversible metabolism (Fig. 30). The pool size of the IAA

**Phenoxy-carboxylic acids and Benzoic acids****2, 4-D****MCPA****Dichloroprop-P****2, 4-DB****Chloramben****Dicamba****Pyridine carboxylic acids****Clopyralid****Picloram****Fluroxypyr****Quinoline carboxylic acids and Others****Quinclorac****Quinmerac****Benazolin****Auxin Transport Inhibitors****Diflufenzopyr-Na****Naptalam****TIBA**

**Figure 29** Structures of selected herbicidal auxins and three auxin transport inhibitors. A total of ca. 30 auxin herbicides are known, of which ca. 20 belong to the phenoxy carboxylic acid structural group.

precursor tryptophan generally is up to three orders of magnitude higher than the IAA pool size. In addition, the pool size of reversible IAA conjugates is one to two orders of magnitude higher. It is therefore important to realize that the response of a tissue to IAA is apparently strongly dependent on the concentration of receptor molecules in that tissue, thereby adding another element of regulation. The hormone action of auxins is connected to the unidirectional (basipetal) transport from leaves to roots, from the synthesizing tissue (leaves = source) to the sinks, which are



**Figure 30** This scheme integrates information on natural and synthetic auxin uptake, metabolism, and biological action. Quite well known are the auxin binding protein 1 (ABP1) on the outer plasma membrane face and the chain of effects leading to the "acid growth" phase: a second messenger sequence leads to proton extrusion and cell wall pH decrease. The auxin permeation through the membrane and the action via a cytoplasmic ABP<sub>c</sub> are less well known. The nuclear induced "synthesis growth" phase could be induced either directly by a G-protein response at the inner plasma membrane face or/and from the ABP<sub>c</sub>. For the in vivo regulation of metabolism and growth, the free auxin pool is in turn regulated by the reversible and irreversible metabolism of the natural auxin indole-3-acetic acid (IAA).

meristems and roots. The auxin permease molecules are therefore concentrated in the plasma membrane on the upper and lower sides of the cells of the conducting tissue (the phloem).

The physiological action of auxins is started by their binding to a receptor and the induction of a sequence of responses leading to the two measurable results of acid growth and synthesis growth (Fig. 30). The best known receptor candidate is the auxin binding protein 1 (ABP1), which may eventually lead to the activation of a proton exporting adenosine

triphosphatase (ATPase) (125). The lowered cell wall pH and activation of polysaccharidases result in weakening of the cell wall and the rapid response, the acid growth phase. The activation of nuclear genes for the specific synthesis of RNA and proteins required in the later but more persistent synthesis growth phase may be triggered directly by G proteins of the G-protein-coupled receptor (GPCR) or/and indirectly by the mediation of cytoplasmic auxin binding protein (ABP<sub>c</sub>). Many auxin binding proteins have been found in different studies, and almost certainly we can anticipate a family of ABPs with different cellular targeting and functions (126–128).

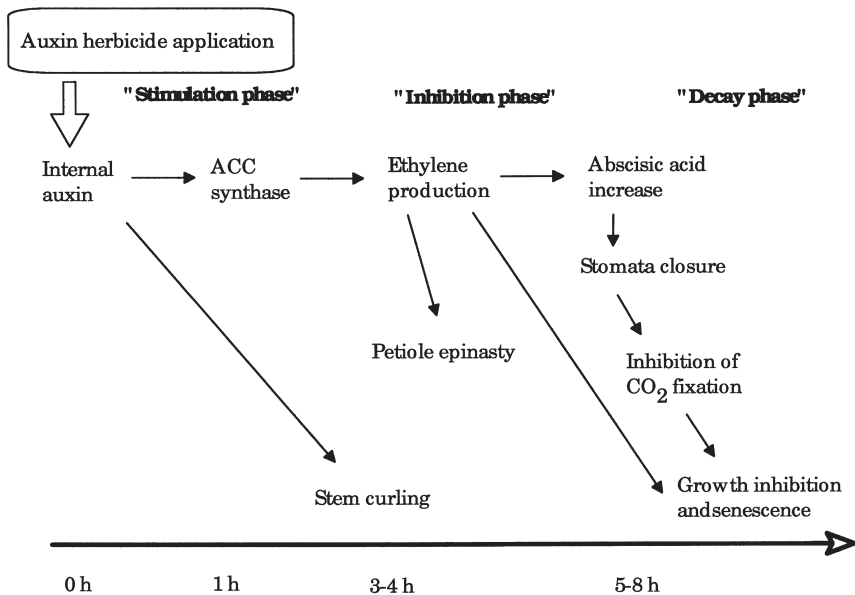
## **B. Affected Metabolism: The Balance of Plant Growth Hormones**

As indicated in Fig. 30, many aspects have to be considered in the effect of auxin herbicides on plant metabolism. The first aspect to know is the concentration of active molecules. The metabolizing reactions regulating the concentration of free IAA are further controlled by other plant hormones, in particular by cytokinins (129). On the contrary, auxin has been shown to promote the biosynthesis of active gibberellin by up-regulating messenger RNA (mRNA) for gibberellin synthesis enzymes (130). Auxin herbicides in principle are subject to the same metabolism as natural IAA. Contrasting with the natural hormone, however, they are often poor substrates for these metabolizing enzymes, with the consequence of slow metabolism and high internal hormone concentrations. These then excessively trigger particularly the stimulation phase and lead to high rates of specific messenger RNA and protein syntheses, which are required for the generation of new growth. These anabolic biosynthetic sequences are supported by the simultaneous mobilization of reserves, by the depolymerization of cell wall xyloglucans and arabinogalactans, and by the stimulation of synthesis of the plant growth hormones ethylene and abscisic acid (131–133).

The common auxin herbicides act only on broadleaf (dicotyledonous) species and are therefore traditionally applied in cereal crops. A different case is realized with quinclorac, which additionally controls some grass species, such as *Echinochloa hispidula* (134, 135). Only susceptible grass species appear to react by high rates of synthesis of the ethylene precursor biosynthetic enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in the roots. Then ACC is transported to the shoot, where it induces more ACC synthesis and its conversion to ethylene and cyanide. The cyanide accumulates up to 30  $\mu$ M and eventually causes the herbicidal action by inhibition of many enzymes, such as cytochrome c oxidase, ribulosebiphosphate carboxylase, and nitrate/nitrite reductase.

### C. Physiological Effects: The Deregulation of Growth

The herbicidal action of artificial auxins can be divided into three phases: (a) stimulation (metabolism and growth), (b) inhibition (metabolism and growth), (c) decay (senescence and breakdown). Although auxin is basically a growth-stimulating hormone, a constant very high concentration in the tissue leads to an imbalance of plant hormones and eventually to the induction of synthesis of the growth inhibitory plant hormone abscisic acid. The stimulation as well as the inhibition phase, therefore, both apply to metabolism as well as growth. The phytotoxic effect of herbicidal auxins is basically caused by their persistently very high concentration in the tissue, with the effect of eventual growth deregulation and inhibition (Fig. 31). High ethylene concentrations stimulate abscisic acid biosynthesis via auxin-induced ACC synthase (136). High abscisic acid concentrations lead to stomata closure and consequently to inhibition of CO<sub>2</sub> assimilation and



**Figure 31** Time course of events leading from herbicide application to growth inhibition and senescence. The stimulation phase includes activation and induction processes for enzymes and metabolites including those for auxin-induced growth ("stem curling") and for the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. ACC is the precursor of ethylene. Ethylene in turn induces abscisic acid synthesis, which induces stomata closure, which leads to inhibition of photosynthesis.

growth (137). Later, abscisic acid, together with ethylene, induces senescence. Transiently, meristematic and cambial cells are growing and dividing to produce new tissue. This growth is not coordinated with the plant's needs and is uneven with respect to the tissue position in the plant. Curling and swelling of tissue therefore occur. In leaf cells, chloroplasts swell and the intracellular compartmentation is gradually lost.

#### **D. Visible Symptoms: From Stem Curling to Tissue Decay**

The auxin herbicide action ends with the decay phase, indicating that the reserves have been used up and the regulation of tissue growth has been completely lost. Before this end, leaves become dark green and wither, stems are curling and twisting, and leaf petioles turn downward, a phenomenon called *epinasty*. The stem tissue stays green longest.

### **VI. INHIBITION OF FATTY ACID AND COFACTOR BIOSYNTHESIS**

#### **A. Inhibition of Acetyl-Coenzyme A Carboxylase**

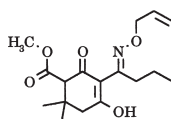
Acetyl-coenzyme A carboxylase (ACCase) catalyzes the first committed step in fatty acid biosynthesis. The inhibitors of ACCase belong to two structural groups. The larger group contains the FOPs, comprising about 30 commercialized compounds, whereas the DIM group contains only ca. 10 compounds developed as herbicides (Fig. 32). In the FOP series, containing substituted propionic acids, of the two enantiomers only the R(+)-isomer is herbicidally active. Only half the dose required of the racemate must therefore be applied of the respective P-labeled active enantiomers. The FOPs are active as the free substituted propionic acids but are applied as esters for better uptake in the standard postemergence applications. Some FOPs are applied with a safener to increase the tolerance to the crop plant, e.g., clodinafop-propargyl (138, 139). All of these ACCase-inhibiting herbicides are only active against grasses and are therefore also called *graminicides*. Some may even be applied in cereals for the control of problem grasses, such as *Avena*, *Bromus*, *Echinochloa*, *Digitaria*, or *Setaria* species. A third group of ACCase inhibitors are the keto-enols, which have not led to a commercial herbicide (140).

##### **1. Primary Target: A Specific Graminicide Target**

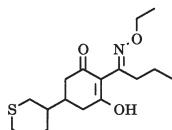
In plants, two different ACCase enzymes are known: a prokaryotic form found in the chloroplasts of dicotyledonous plants and a eukaryotic form in



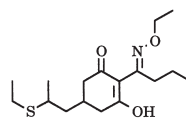
## Cyclohexanediones (DIMs)



Alloxydim

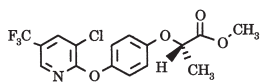


Cycloxydim

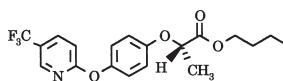


Sethoxydim

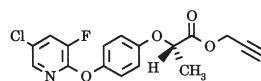
## Aryloxyphenoxy-propionates (FOPs)



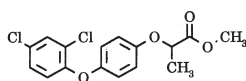
Haloxfop-P-methyl



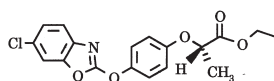
Fluazifop-P-butyl



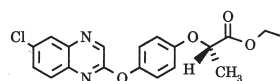
Clodinafop-propargyl



Diclofop-methyl



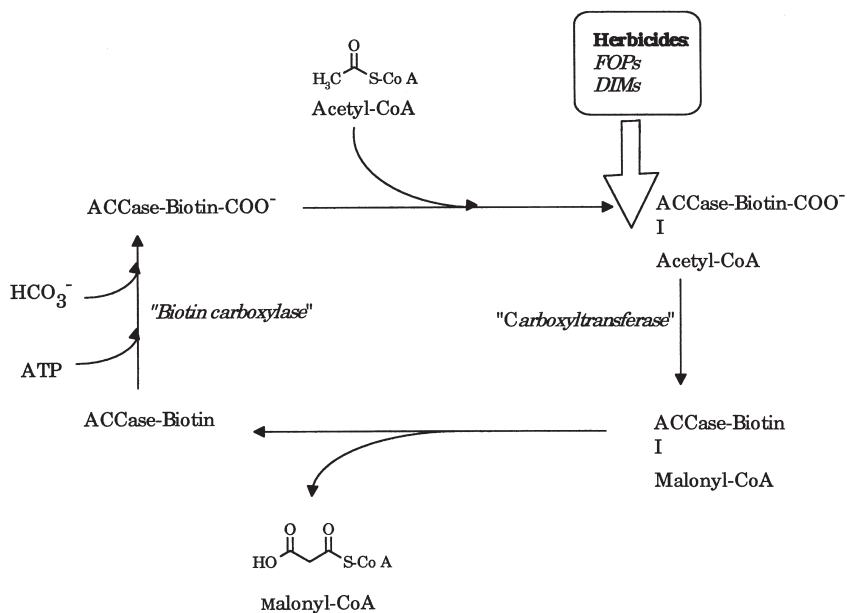
Fenoxaprop-P-ethyl



Quizalofop-P-ethyl

**Figure 32** Structure examples of herbicidal inhibitors of acetyl-coenzyme A carboxylase (ACCase).

the cytoplasm of all plants, but also in the chloroplasts of grasses (family Gramineae). Whereas the prokaryotic ACCase consists of separate subunits for the partial reactions and also a separate biotin carboxylcarrier protein (BCCP), the eukaryotic multifunctional enzyme (Fig. 33) has all these functions concentrated in one large protein (141, 142). As we can now reconstruct, at one point in the Gramineae evolution, the chloroplast-encoded prokaryotic ACCase multienzyme complex was lost. Instead, a second nuclear-encoded, eukaryotic-type isoenzyme with a transit peptide for chloroplast targeting was developed or, more probably, had already been developed at that point. In principle, only the eukaryotic enzyme is sensitive to the herbicides of the FOP or DIM groups. This enzyme consists of two large subunits, each with 200- to 240-kd molecular weight, and sequences for the partial reactions of the biotin carboxylase, carboxyl-transferase, and specific biotin binding (biotinylated domain) (143). The herbicides bind to the carboxylated protein and inhibit the binding of acetyl-CoA in a noncompetitive manner (144). However, the herbicide sensitivity of different eukaryotic ACCase enzymes of different origins may differ considerably (145). In grasses, the cytoplasmic isoform seems often to be insensitive, whereas the chloroplastic isoform is sensitive. However, in



**Figure 33** Catalytic cycle of acetyl-coenzyme A carboxylation in the multifunctional enzyme. In a first energy consuming step (biotin carboxylase partial reaction) bicarbonate is bound to the enzyme cofactor biotin. Second, acetyl-CoA is bound, and then the carboxylic acid group is transferred to yield malonyl-CoA by the carboxyltransferase partial reaction. The herbicides interfere with the carboxylated enzyme at the binding site for acetyl-CoA/malonyl-CoA and overlapping biotin binding site. They may act as transfer complex analogs. FOPs, aryloxyphenoxypropionates; DIMS, cyclohexanediones; ACCase; acetyl-coenzyme A carboxylase; CoA, coenzyme A.

dicotyledonous weeds the cytoplasmic multifunctional isoform is often sensitive.

Many resistant grasses in which the chloroplast isoenzyme has become resistant are known, but also increased metabolism is often the resistance mechanism (146). The Herbicide Resistance Action Committee has published two reviews of graminicide resistance and cross-resistance (2). Large differences in resistance may exist with different herbicides and enzymes from even closely related resistant plant species. The sensitivities are not the same in similar isoenzymes from different resistant plants. Thus, considerable variation exists between homologous enzymes from different plant species. In the native dimer of less herbicide-sensitive or -resistant ACCase isoenzymes, the binding of the second graminicide molecule occurs with a

lower affinity than the binding of the first graminicide molecule (144). It therefore appears that cooperativity of herbicide binding may be associated with low sensitivity of the enzyme.

## 2. Affected Metabolism: Cooperation of Chloroplasts and Cytoplasm

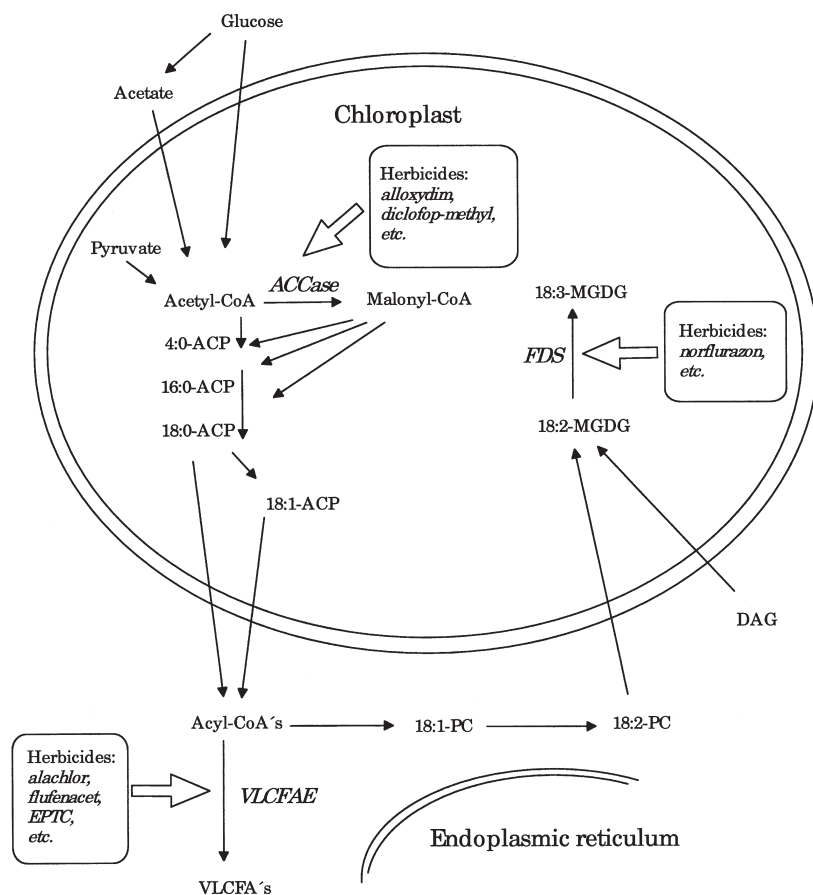
Fatty acid biosynthesis in plant cells occurs nearly exclusively in chloroplasts (Fig. 34). In nongreen tissue, low rates are possible in leukoplasts. The starting reaction is the activation of the C2 building block acetyl-CoA by carboxylation to malonyl-CoA, the target of the herbicides dealt with in this chapter. By successive addition of C2 from malonyl-CoA, different fatty acid synthetases synthesize chain lengths up to C18:0. During this synthesis sequence the growing fatty acid chain is bound to the respective synthetase complex via an acyl-carrier protein (ACP).

In chloroplast thylakoid membranes the main lipids are mono- and digalactosyl diglycerates (MGDGs and DGDGs), which contain high amounts of 18:2 and 18:3. The desaturation from 18:1 to 18:2 occurs outside the chloroplast at the endoplasmic reticulum. To this end, 18:1 is transported outside the chloroplast as the CoA ester and incorporated into phosphatidylcholine (PC). Other diacylglycerols (DAGs) can enter the chloroplast and galactolipid pool. Synthesis of the very-long-chain fatty acids (VLCFAs) from 18:0-CoA to chain lengths in excess of C30 also occurs at the endoplasmic reticulum, with cytoplasmic malonyl-CoA as the second substrate.

## 3. Physiological Effects and Visible Symptoms: A Case for Meristems

Fatty acids are main components of all cellular membranes. Commonly, glycerol is diacylated with two more or less unsaturated fatty acids of C18 chain length. The third position carries a component that can be, among others, mainly choline, phosphatidyl ethanolamine, or inositol in cytoplasmic membranes and (di)galactose in chloroplast membranes. Since the fatty acids of all membranes are synthesized in the chloroplast, an inhibition of chloroplast ACCase blocks all cellular fatty acid synthesis and therefore inhibits the growth of all cellular membranes, e.g., plasmalemma, tonoplast, endoplasmic reticulum, and, of course, also chloroplast membranes. Since the main sites of synthesis of new membranes are in the meristems and the adjacent elongation and growth regions, meristems are particularly sensitive to inhibition by ACCase-inhibiting herbicides.

When only cytoplasmic ACCase is inhibited, there are no obvious herbicidal consequences. Cytoplasmic malonyl-CoA is required for



**Figure 34** Biosynthetic pathways for long-chain ( $\leq 18:0$  saturated carbon chain length), and very-long-chain fatty acids (VLCFAs:  $\geq 20:0$  saturated chain length) and their desaturations to oleic (18:1), linolenic (18:2), and linoleic (18:3) acids. This scheme shows a simplified selection of the pathways important for herbicide modes of action in typical “18:3” plants (147–149). Herbicides and their target enzymes are indicated by open arrows. The target enzymes shown here are ACCase, very-long-chain fatty acid elongases (VLCFAEs, Sec. VI.B), and fatty acid desaturases (FDSs, Sec. III.A.2).

polyketide and flavonoid biosynthesis, for malonylation as a final step in the degradation of xenobiotic compounds, and for fatty acid elongation to VLCFAs. The latter reaction is the primary target of many diverse herbicides and is dealt with in the next section.

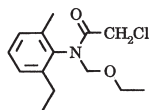
Established leaf tissue is not particularly sensitive to ACCase herbicides. Still, the inhibition of fatty acid synthesis in isolated chloroplasts in the light is very sensitive. The  $I_{50}$  concentrations are generally between 20 nM for the highly inhibitory FOPs (e.g., quizalofop) and 7  $\mu$ M for the less inhibitory DIMs (e.g., sethoxydim). The turnover of fatty acids in leaf tissue is obviously too low to allow a shortage to occur. However, the very young and actively growing leaf bases turn light green and their tissue is destroyed after herbicide application. This effect causes the upper part of the grass plant to be easily separated when twisted in its meristematic base. To understand this we have to keep in mind that grasses contain their meristems at the base of the plant, hidden in the leaf sheaths of the older leaves. When these meristems are severely damaged, the old leaf parts are then cut off from their supporting base.

At the cellular level, fatty acid biosynthesis is completely inhibited after 1 h in root meristems. After 2 h, DNA and cell wall synthesis are stopped, and after 4 h all growth ceases. After 24 h, the cytoplasm is vacuolized, and after 48 h mitotic figures and nuclei disappear. In contrast, the older parts of the roots are insensitive.

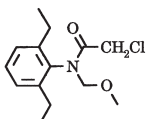
## B. Inhibition of Very-Long-Chain Fatty Acid Elongases

Figure 35 contains herbicides from eight different structure groups and some additional structures that appear to inhibit elongases for very-long-chain fatty acids (VLCFAs). Only a few examples are shown here from the large groups of  $\alpha$ -chloroacetanilides (ca. 30 commercial  $\alpha$ -chloroacetamides are known, of which most are  $\alpha$ -chloroacetanilides; nonaromatic chloroacetamides are not shown here) and thiocarbamates (ca. 20 have been commercialized). These molecules have in common a reactivity as an alkylating agent by an electrophilic attack, with  $\text{Cl}^-$  (chloroacetamides),  $\text{R}-\text{O}^-$  (oxyacetamides), or  $\text{R}-\text{S}^-$  (phosphothioacetamides) acting as leaving groups. The target molecule thereby becomes substituted with the acetamide part of the herbicide. It was originally speculated that the mode of action might include alkylation at the target enzyme, specifically at an enzyme SH-group, but the concentrations required for alkylation (100  $\mu$ M) are far in excess of those for herbicidal action (1  $\mu$ M).

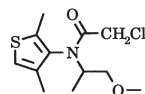
The facile reaction with nucleophiles is realized with glutathione (GSH). The  $\text{GS}^-$  replaces the leaving groups named in the herbicide molecules and thereby detoxifies them. In the case of the thiocarbamates, the molecule must first be activated to the sulfoxide by oxidation with monooxygenases. The sulfoxide part is then replaced by  $\text{GS}^-$ . The reaction is catalyzed by glutathione-S-transferases (GSTs) and constitutes an

**$\alpha$ -Chloroacetanilides**

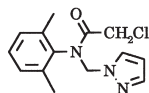
Acetochlor



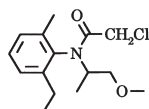
Alachlor



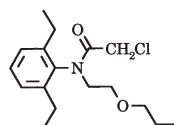
Dimethenamid



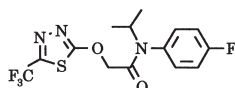
Metazachlor



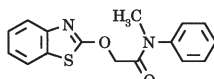
Metolachlor



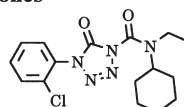
Pretilachlor

**Oxyacetamides and Tetrazolinones**

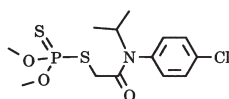
Flufenacet



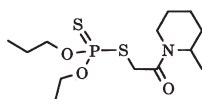
Mefenacet



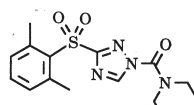
Fentrazamide

**Phosphothioacetamides and Others**

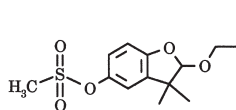
Anilofos



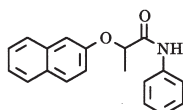
Piperophos



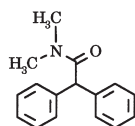
Cafenstrole

**Benzofuranes and Acetamides**

Ethofumesate



Naproanilid

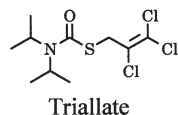
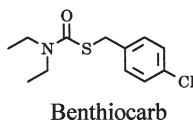
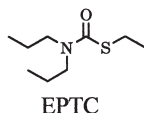


Diphenamid

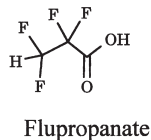
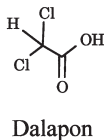
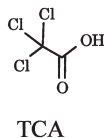
**Figure 35** Structural examples of herbicides inhibiting lipid biosynthesis at the elongases for very-long-chain fatty acid (VLCFA) biosynthesis.

important detoxification mechanism for all these herbicides, particularly in corn (maize). In corn and some other crop species specific GSTs are additionally induced by safeners, which are therefore added to increase the crop tolerance of many of these herbicides (see Chapter 9 for details).

### Thiocarbamates



### Halogenated Carbonic Acids



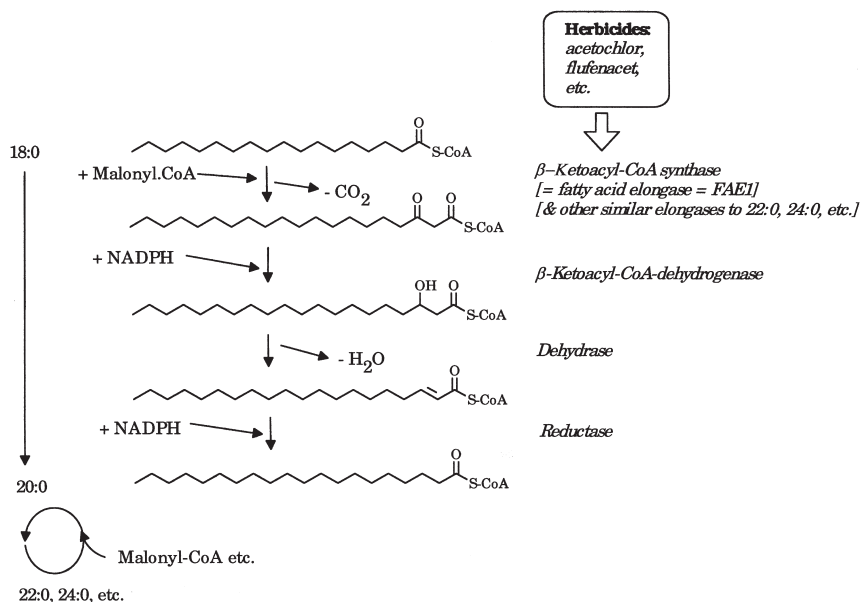
**Figure 35** Continued.

Benzofuranes, acetamides, and halogenated carbonic acids have long been known as inhibitors of lipid biosynthesis, cell division, and growth. However, since the very-long-chain fatty acid elongases (VLCFAEs) as primary target enzymes have not been intensively studied and have only recently been clearly recognized as the primary target of  $\alpha$ -chloroacetanilides, the inclusion of compounds such as the benzofuranes is to some extent preliminary. It is based on structure similarities with the essential structural elements and on earlier data on the inhibition of lipid biosynthesis (150). Also, the herbicidal effects, including interference with safeners, are very similar.

The herbicides in Fig. 35 act on grasses and broadleaf weeds, but the activity against grasses is much stronger, especially for the thiocarbamates. They are applied preemergence and act particularly on germinating grass seedlings.

#### 1. Primary Target, Affected Metabolism, and Physiological Effects: Membrane Structure and Evaporation Protection

Figure 34 gives an overview of the biosynthetic pathways leading to long-chain and very-long-chain fatty acids in plant tissues. Whereas the chloroplast is the only important subcellular site for long-chain fatty acid biosynthesis, the VLCFA biosynthesis occurs at the endoplasmic reticulum. From here the VLCFAs are transported to the growing membranes and to the leaf surface, where they are oxidized, esterified, and polymerized to yield the water-protecting internal and external layers, e.g., cutin, suberin, and waxes. Figure 36 shows a VLCFA elongation cycle. The elongases are



**Figure 36** Elongase systems for the elongation of fatty acids with chain lengths  $\geq$  C18 in the cytoplasm and endoplasmic reticulum (compare Fig. 34). The rate of an elongation cycle is controlled by the activity of the respective  $\beta$ -ketoacyl-coenzyme A synthase for the elongation step. As a group, these elongase enzymes are called *very-long-chain fatty acid elongases* (VLCFAEs). The three other enzymes (written in *italic*) required for the dehydrogenations and the dehydration appear to be unspecific and are not rate-limiting. The herbicides listed in Fig. 35 appear to inhibit different elongases for different chain lengths and different fatty acid substrates differently. Details of the elongases for different metabolic functions are largely unknown. CoA, coenzyme A.

progressively inhibited with growing chain length by chloroacetanilides, oxyacetamides, tetrazolinones, cafenstrole, etc. (compounds of Fig. 35) (151, 152). The increase of the sensitivity of a microsomal *in vitro* elongation system from *Allium porrum* toward metazachlor with growing chain length ranged from  $I_{50} = 1 \mu\text{M}$  for 20:0 to  $I_{50} = 10 \text{ nM}$  for 24:0 synthesis.

The VLCFAs are regular constituents of plasma membranes at very low concentrations. They appear to be required for membrane curvatures in all organelles and for all types of membranes (153). An overexpression of fatty acid elongase 1 (FAE1) in *Arabidopsis* sp., leading to very high amounts of VLCFAs in all major membrane lipid classes, severely



inhibited growth of the whole plant. Conversely, a lack of VLCFAs apparently also severely inhibits meristem and tissue growth, because normal membrane growth is no longer possible. It was very early noted that application of  $\alpha$ -chloroacetanilides leads to a rapid loss of membrane semipermeability. On a cellular level, both cell growth and entry into cell division are inhibited (154). The many studies on  $\alpha$ -chloroacetanilide effects on cellular growth and metabolism have been summarized repeatedly (155, 156). Strong effects on lipid spectra have been noticed in these experiments. Other data were largely inconclusive.

For thiocarbamates and some other structures, such as ethofumesate, however, VLCFA synthesis was early recognized as a mode of herbicide action. These data were on effects on chain length in extracellular lipids, but decisive *in vitro* studies with the enzymes described could not be performed for a long time. The situation with the thiocarbamates is further complicated by the fact that they apparently must be activated to the sulfoxide, which is rapidly further metabolized to the sulfone and to glutathione conjugates. The actual level of active herbicide in the tissue is therefore not known, and in many physiological experiments a lack of metabolism has probably hindered the studies.

An effect of all the herbicides discussed here is on the synthesis of wax, cutin, and suberin layers on the leaf and stem surfaces. The amount of these evaporation-reducing layers and compounds is strongly reduced and their composition is changed. In view of our present knowledge this is not surprising, since these lipophilic structure classes contain very-long-chain carbon components such as acids, aldehydes, alcohols, ketones, and esters, singly or reacted to complex polymers (157). For the synthesis of these structural classes, the VLCFAs may be oxidized at different positions in the carbon chain or decarboxylated to alkanes to form the great variety of lipophilic molecules mentioned. As a result, these herbicides lead to much decreased outer lipophilic layers on all aerial plant surfaces, and to significantly increased rates of cuticular water evaporation. Differences among different herbicide structural groups in their precise physiological effects may be related to the differential inhibition of specific VLCFA elongases for different metabolic functions.

## 2. Visible Symptoms

The visible symptoms in grass seedlings for the structures in Fig. 35 include decreased growth and leaf curling and twisting. The leaf tip often remains attached to the coleoptile tip, and the growing leaf therefore tends to bend out of the ruptured coleoptile and form a loop structure. Since only the meristems and adjacent elongation zones at the leaf base are

sensitive, dark green leaf tissue grows out of the disturbed plant base but remains short, twisted, and strongly inhibited. The complete elimination of these effects by safeners (Chapter 9) is always very impressive and can be taken as a group characteristic of these herbicides. Halogenated carbonic acids differ somewhat in that the safening action is not so clearly documented.

## VII. CELL WALL TARGETS

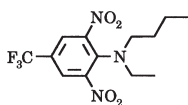
### A. Interference with Microtubular Systems

The herbicides known to interfere with the microtubular system of plant cells (also called *mitotic disrupter herbicides*) are structurally very diverse (Fig. 37). They are primarily graminicides that can be applied preemergence in all dicotyledonous crops, some even in cereals. The dinitroanilines form the largest group (14 commercialized compounds are known). Some dinitroanilines have high vapor pressure, which indicates that they have to be incorporated into the soil to obtain full herbicidal activity. CIPC (chlorpropham) is applied on potato tubers to prevent sprouting. Amiprofos-methyl and dithiopyr may be used to control grasses in turf (golf). Chlorthal-dimethyl is also known as *DCPA*, and pronamide as *propyzamide*.

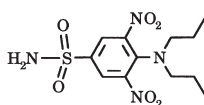
#### 1. Primary Targets: Tubulin and Microtubule Organizing Centers

The globular proteins  $\alpha$ - and  $\beta$ -tubulin, after dimerizing into tubulin, aggregate spontaneously into microtubules at different cell locations when certain conditions are met (Fig. 38). The microtubules are hollow, rigid cylinders formed by the longitudinal aggregation of tubulin dimers into 13 parallel protofilaments. The aggregation is controlled by different microtubule organizing centers (MTOCs) in different locations of the cell and by the local guanosine triphosphate (GTP) availability and low  $\text{Ca}^{2+}$  concentrations. This means that microtubules are constantly assembled at one end and disassembled at the other end, a process called *treadmilling*. Additionally different microtubule-associated proteins (MAPs) participate in the polymerization into microtubules of different functions. Although different  $\alpha$ - and  $\beta$ -tubulins occur for possibly different functions, the pools of free  $\alpha$ - and  $\beta$ -tubulins/tubulin heterodimers are used again and again in the reversible tubulin polymerizations, producing microtubular polymerization cycles at different locations and for different functions during the cell cycle.

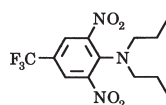
### Dinitroanilines



Butralin

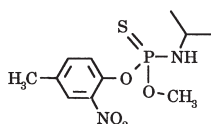


Oryzalin

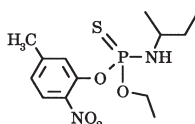


Trifluralin

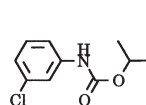
### Phosphoroamidates, Carbamates and Pyridines



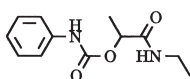
Amiprofos-methyl



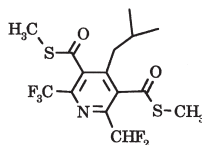
Butamiphos



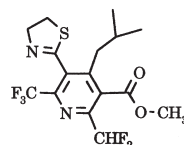
CIPC



Carbetamide

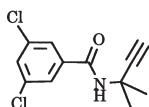


Dithiopyr

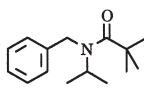


Thiazopyr

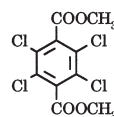
### Benzamides and Benzoic acids



Pronamide



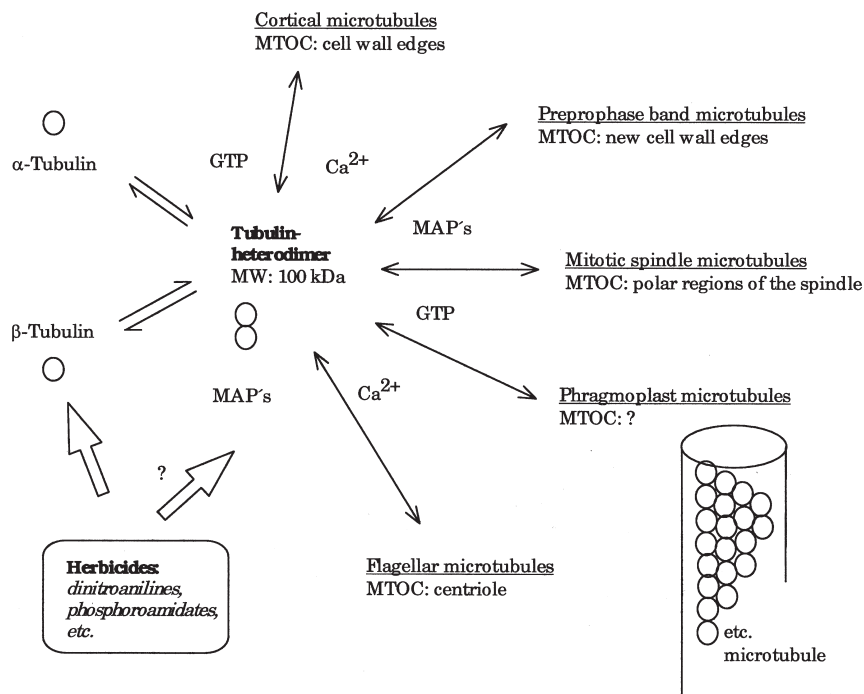
Tebutam



Chlorthal-dimethyl

**Figure 37** Herbicides that interfere with the microtubular system in plant cells.

During interphase, cortical microtubules are nucleated at the cell edges. These cortical microtubules are located close to the plasmalemma and are connected to it by “bridges.” They control the direction of cellulose microfibril deposition during cellulose biosynthesis and thereby indirectly determine the strength of the cell wall. They regularly change their direction by 60°–90°, producing crossing layers of cellulose microfibrils in the growing wall. During preparation for cell division, a preprophase band of microtubules runs around the cell wall, marking the new division plane. Next, the tubulin rearranges into the spindle microtubules that serve in



**Figure 38** Reversible polymerization of tubulin into functionally different microtubules. The polymerization process is controlled by guanosine triphosphate (GTP), the  $\text{Ca}^{2+}$  concentration, additional microtubule-associated proteins (MAPs), and the activity of different microtubule organizing centers (MTOCs).

chromosome distribution between the daughter cells. Finally during telophase, the phragmoplast constitutes two microtubular rings of opposite polarity in the anticipated plane of the new cell wall. These attract vesicles with the materials for the new wall. The new cell wall grows outward from the original central cell plate until reaching the existing cell walls, separating the daughter cells after division. The MTOCs may only be seen as dark areas in electron micrographs and are often very difficult to visualize at all.

Whereas the microtubules described so far are of the labile type, flagellar microtubules are stable, i.e., they do not participate in the regular cell cycle. Higher plant cells do not contain flagellae or cilia in any phase of their life. However, the growth of algal flagellae is strongly inhibited by all herbicides of this group. For example, the green alga *Chlamydomonas reinhardtii* may be induced to shed its flagella by vigorous shaking of

the medium at pH 5. The regrowth of a new flagella, which occurs within 90 min in untreated cells, is strongly inhibited by the herbicides discussed here (158, 159).

The herbicides shown in Fig. 37 interfere either with tubulin polymerization or with the MTOC, leading to loss of microtubules (dinitroanilines and phosphoroamidates) or to disorientation of microtubules that do not function properly (carbamates). Dinitroaniline herbicides have been shown to bind to isolated tubulin (160). Colchicine, a well-known natural cell division inhibitor, binds to  $\beta$ -tubulin. Since colchicine-resistant mutants of *Chlamydomonas reinhardtii* are also resistant to dinitroanilines and phosphoroamidates, these should also bind to  $\beta$ -tubulin. Cross-resistance phenomena in resistant *Eleusine indica* and *Setaria viridis* have also demonstrated similar binding for dinitroanilines and phosphoroamidates. Interestingly, the resistant lines were “super-sensitive” to CIPC (161, 162). Binding of colchicine or herbicides leads to inhibition of tubulin polymerization, resulting in the loss of microtubules. The anticancer compound taxol causes a higher than normal stability of microtubules, inhibiting treadmilling and thereby also the normal function of the microtubular cycle. Herbicides, however, have not been shown to stabilize existing microtubules. It is not clear, besides the probable binding to  $\beta$ -tubulin of some compounds, what the precise interference point is for the different herbicides, sometimes even from the same structural group. Individual carbamates and dinitroanilines lead to loss of microtubules or, in some cases, only to disorganization of microtubules (163, 164). Pyridines may bind to specific MAPs (165, 166).

## 2. Affected Metabolism and Physiological Effects: Cytoskeleton and Cell Wall Dysfunctions

As may be deduced from the interference points discussed, microtubule disrupter herbicides interfere with cell division and cell wall growth. Cell division is rapidly and reversibly blocked at metaphase after 30–60 min. Cells in metaphase accumulate with time in treated tissue. This accumulation is the consequence of lacking a functional mitotic spindle body. The loss of spindle microtubules may be observed after 5–10 min. Since the cortical microtubules are also missing or are disoriented, secondary wall thickening does not occur. The new cell walls remain thin and cannot resist the cell turgor, growing to very large cells with several times the volume of normal cells. Such cells are isodiametric and do not elongate in the direction of the growing plant organ. The divided chromosomes accumulate in the growing cells in very large nuclei. Obviously, a new nuclear membrane is formed around the daughter chromosomes. Also, micronuclei and polymorphic

nuclei have been observed. The action of mitotic disrupter herbicides has been described in detail by Hess (167).

A new addition to the list of effects concerns stomatal functioning. This may not be relevant to normal herbicide action during seedling growth, but learning that microtubule disrupter herbicides inhibit stomatal opening is interesting (168). This is an example of the many cases in which herbicides were used in basic plant research as specific metabolic inhibitors. The inhibition of stomatal opening seems to be related to microtubule function upstream to the control of ionic fluxes leading to stomatal opening, perhaps via its association with MAPs.

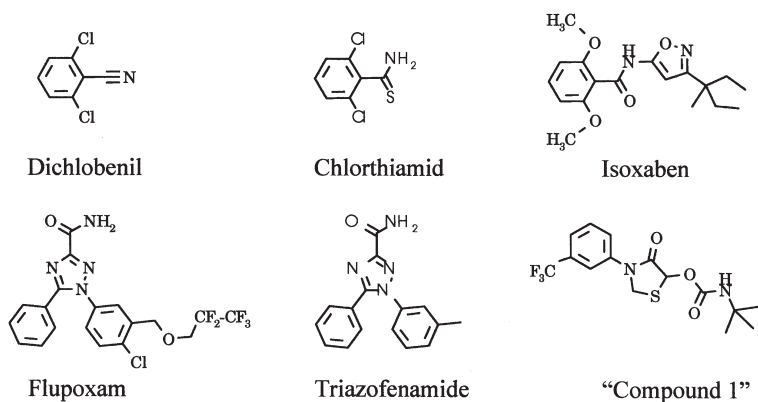
Only after several hours to days are other metabolic processes inhibited indirectly. Synthesis of DNA, RNA, and protein is inhibited after 5–7 days. At increased concentrations (10–100  $\mu$ M), uncoupling was reported. Trifluralin also inhibits the reoxidation of reduced plastoquinone in photosynthetic electron transport. However, these observations have no bearing on the herbicide mode of action under standard preemergence application conditions.

### 3. Visible Symptoms: Enlarged Cells and Swelling Tissues

Large, isodiametric giant cells are commonly found in the root or shoot elongation zone of plants treated with herbicides that interact with tubulin. Their presence leads to tissue swelling, especially in roots, which appear club-shaped at their tips. Depending on the function of the microtubules in the growth of a particular tissue, the morphological appearance may vary. Branched root hairs have been observed. In conducting tissue, the secondary wall thickening required for a functional xylem is not formed, resulting in weakening of the stem. The young seedlings are not supplied by water and salts from root uptake, and very little growth occurs above the soil. If growth occurs at sublethal concentrations, secondary “crown” roots in Gramineae (e.g., in maize) are not formed. Such plants fall over (lodge) in wind or rain.

## B. Inhibition of Cellulose Biosynthesis

Of the herbicides shown in Fig. 39, dichlobenil and chlorthiamid are nonselective. Chlorthiamid is metabolized to dichlobenil in the soil. The other compounds are selective in cereals or maize (flupoxam). Triazofenamid (169) and Compound 1 are recent additions to the list of cellulose synthesis-inhibiting herbicides. Compound 1 (170) is an experimental herbicide added to this list because of its new chemical structure and some observations suggesting inhibition of cellulose biosynthesis as the mode of action: (a) symptoms, (b) specific inhibition of  $^3\text{H}$ -glucose incorporation



**Figure 39** Herbicides known to inhibit cellulose biosynthesis.

into the cell wall fraction containing cellulose, and (c) cross-resistance with an isoxaben-resistant *Arabidopsis thaliana* selection.

### 1. Primary Target: A Complex Complex

Cellulose synthase is an oligomeric assembly of six subunits forming a "rosette," a membrane-integrated protein complex for the synthesis of cellulose microfibrils. The rosettes move in the plane of the plasma membrane and are guided by microtubules that are located 20 nm below the plasma membrane at 40-nm intervals, thereby producing layers of parallel cellulose microfibrils on the inner side of the growing cell wall (171, 172). The herbicides in Fig. 39 appear to inhibit the synthesis of the cellulose microfibrils, but precisely where they interfere is not known. Details of the cellulose synthase complex are unknown, and it seems probable that several different molecular interference sites for the different herbicide structures are involved.

Diclobenil binds to an 18-KDA polypeptide with unknown function in cotton fibers. Other cellulose synthesis inhibitors, such as an experimental thiazotriazine herbicide, cause a disintegration of rosette structures (171, 173). The enzyme activity obviously is not rapidly inhibited, leading to the accumulation of noncrystalline glucans in this case. Still other inhibitors lead to a very rapid inhibition of cellulose synthesis within minutes (170). In recent years, a cellulose synthase superfamily of genes that should greatly help in the elucidation of the details of the cellulose synthase complex has become known (174). The problem of in vitro testing of cellulose synthase may then be solved in the near future. So far, cellulose synthase is active only under in vivo conditions.

## 2. Affected Metabolism, Physiological Effects, and Visible Symptoms: Severe Growth Inhibition

Isoxaben, triazofenamide, and, to a lesser extent, dichlobenil ( $I_{50}$  1, 39, and 400 nM) are potent inhibitors of the incorporation of  $^{14}\text{C}$ -glucose into the cellulose-containing cell wall fraction of *Arabidopsis thaliana* (169). Root tips of treated *Arabidopsis thaliana* plants are approximately two fold greater in diameter when compared to the controls, and root hairs continue to differentiate on the root epidermis, so that they appear to cover the root tips. This combination of symptoms is indicative of cellulose biosynthesis inhibitors, whereas after treatment with microtubule disrupter herbicides, the roots are larger in diameter and do not form root hairs (175). Also, microtubule disrupter herbicides act specifically against grasses and show very little activity against dicotyledonous plants. The symptoms described and severe stunting occur at low to intermediate rates. At higher rates, seed germination is completely inhibited. In the sublethal range, swelling and splitting of the stems occur, in addition to stunting, swelling, and browning of roots.

The inhibition of cellulose synthesis can also be visualized microfluorimetrically by the specific cellulose complexing calcofluor white in cell protoplast suspensions (176). In the presence of dichlobenil, the common cellulose-xyloglucan cell wall is no longer present. Instead, the rudimentary "replacement" cell wall regenerated by the protoplasts contains more galacturonic acid polymers.

Inhibitors of cellulose biosynthesis have little or no inhibitory action on other metabolic processes of plants. Protein, nucleic acid, and lipid metabolism are not affected rapidly at micromolar concentrations. Only after the inhibition of growth are these pathways inhibited secondarily. Dichlobenil, after hydroxylation in the 3- and 4-positions, is also a respiratory uncoupler (177). However, greater than herbicidal concentrations (15–62  $\mu\text{M}$ ) are required for this uncoupling activity.

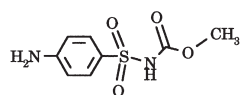
## VIII. SPECIALTIES AND UNKNOWN

### A. Asulam: An Inhibitor of 7,8-Dihydropteroate Synthase

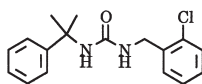
Asulam (Fig. 40) is basically a nonselective herbicide applied for special uses, such as the control of *Rumex* species in pastures or for the control of ferns in forests and forest plantations. Asulam is a carbamate herbicide and may be compared in this respect with carbamate inhibitors of the MTOC (see Sec. VII.A.1). However, asulam has become known to inhibit



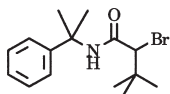
### Asulam and Dimethylbenzylamides



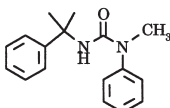
Asulam



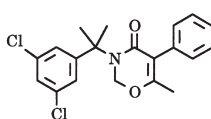
Cumyluron



Bromobutide



Methyl-dymron



Oxaziclomefone

**Figure 40** The herbicides shown here are less well known mechanistically or represent a unique target site (asulam). Asulam inhibits dihydropteroate synthase in folic acid biosynthesis; the other four herbicides are less well studied.

7,8-dihydropteroate synthase (DHPS), an enzyme with an important function in the biosynthesis of folic acid. Folic acid is, in turn, required in C1 metabolism, more specifically for the transfer of activated formic acid and activated formaldehyde between different molecules, connecting anabolic with catabolic pathways. In folic acid biosynthesis, the DHPS substrate, 2-amino-4-hydroxy-6-hydroxymethylpyrophosphoryl dihydropteridine, is condensed with *p*-aminobenzoate to form the 7,8-dihydropteroate product. Other inhibitors of DHPS, e.g., sulfadiazine or sulfanilamide (178–180), do not act as herbicides. The DHPS substrate, *p*-aminobenzoate, antagonizes the herbicidal action of asulam. The herbicidal symptoms have been interpreted to reflect the inhibition of DHPS but not of microtubule function (181–183). Asulam has, however, also been reported to be a mitotic inhibitor in *Allium cepa* (184). Depending on the application situation and treated species, both mechanisms may contribute to herbicide action and symptoms. The symptoms are those of cell division inhibition and mitotic arrest.

### B. Dimethylbenzylamides: Herbicides for Rice

The dimethylbenzylamides are graminicides for use mainly in rice. Although several herbicides of this structural group are known (Fig. 40), a molecular mode of action has not been found so far. The essential element appears to be the isopropane amide structure bound to an aromatic ring, but in the periphery much structural variation is obviously possible. The symptoms in

sensitive plants are similar to those of thiocarbamates (see Sec. VI.B). Safening has been reported between the sulfonylurea herbicide bensulfuron-methyl and dymron or the thiocarbamate dimepiperate. These interesting graminicides may therefore be mechanistically related to the very-long-chain fatty acid elongase (VLCFAE) inhibitor herbicides presented in Sec. VI.B, but so far experimental evidence of direct biochemical effects has not been reported.

## ACKNOWLEDGMENTS

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# 8

## Molecular Basis of Toxic Effects: Inhibition of Cellular Pathways and Structural Components

**K. Kramer and Bertold Hock**

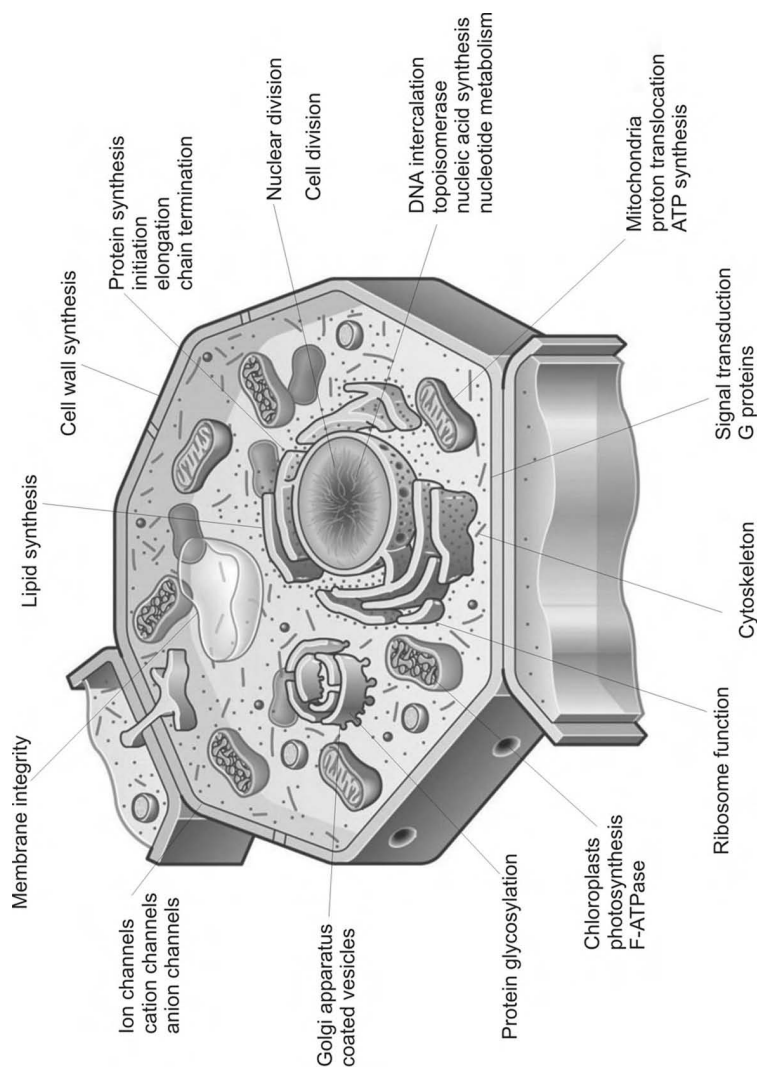
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### I. INTRODUCTION

Plants and fungi have a number of unique metabolic and developmental characteristics. Key reactions involved in those processes can often be identified by selective and carefully characterized inhibitors. An illustrative example is the involvement of G proteins in phytochrome-directed responses that are abolished in the presence of inhibitors such as cholera toxin that knock out the function of G protein. The main emphasis of this chapter is targeted at the molecular mechanism of antibiotic and synthetic compound interference with cellular components and pathways of plants and fungi. The topics include inhibition of nucleic acid synthesis, ribosome structure, and protein and lipid synthesis. Special attention is given to the interference with membranes, ion channels, cytoskeleton, cell wall, cell division, endoplasmatic reticulum, Golgi apparatus, mitochondria, and chloroplasts (Fig. 1). Inhibitors such as herbicides and fungicides are treated in Chapter 7.

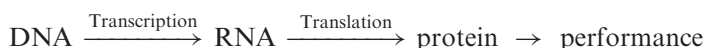
### II. INHIBITION OF NUCLEIC ACID AND PROTEIN SYNTHESIS

Genetic information is encoded by the nucleotide sequence of the deoxyribonucleic acid (DNA). The conversion into the amino acid sequence



**Figure 1** Target sites of inhibitors in plant cells. DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; F-ATPase, (Adapted from [www.oup.co.uk/images/oxed/children/voes/nature/plantcell.jp](http://www.oup.co.uk/images/oxed/children/voes/nature/plantcell.jp).)

of proteins, which determines the characteristics of the cell, is carried out in two steps: transcription and translation.



In addition to its role as an information source, DNA has the capacity of identical reproduction. This is an essential prerequisite for inheritance. The mechanism of DNA synthesis is described as replication. The main part of DNA is found in the nucleus of the eukaryotic cell. Replication and transcription take place in this compartment, whereas translation is carried out in the cytosol. Mitochondria and plastids also contain DNA together with an own-protein synthesis apparatus.

Nuclear nucleic acid synthesis and protein synthesis differ in numerous details from the corresponding processes in mitochondria and plastids. This is explained by the symbiont theory of mitochondrial and plastid evolution. According to this theory mitochondria and plastids are derived from prokaryotic organisms (bacteria and cyanobacteria), which merged with prototypes of eukaryotic protozoa and were domesticated.

### A. Interference with Nucleic Acid Functions

A selection of compounds that are frequently used in plant molecular biology is discussed in the following. These inhibitors block polymerization reactions and interfere with the synthesis of nucleic acid precursors (nucleotides) or the conformation of the DNA double helix.

### B. Intercalating Compounds

Several compounds form complexes with the DNA double helix by intercalating between two successive base pairs. The structural integrity of DNA is not affected by this intercalation.

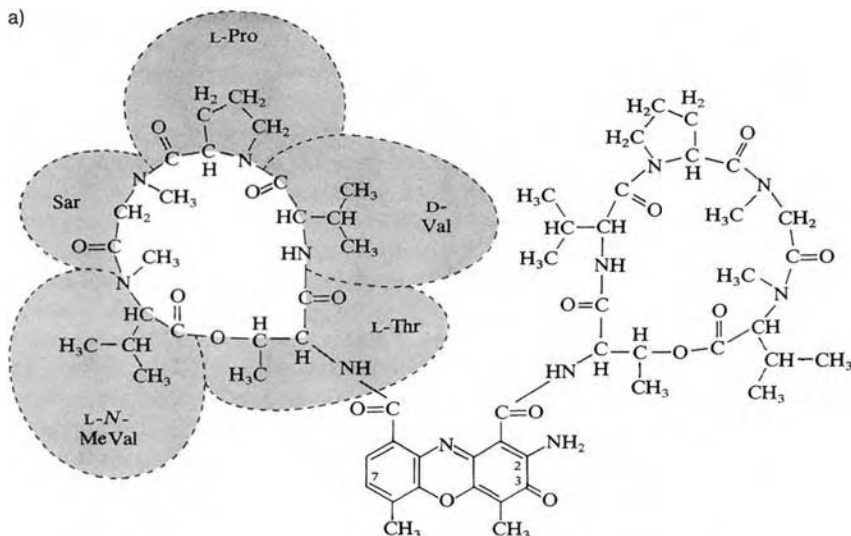
#### 1. Actinomycin D

Actinomycins belong to the polypeptide antibiotics, which were isolated from the actinomycete *Streptomyces antibioticus* already in 1940. The mode of action is due to the specific binding to the DNA double helix, impeding DNA-dependent ribonucleic acid (RNA) synthesis.

Figure 2a shows the best known representative of these compounds, actinomycin D ( $=C_1$ ). Two identical pentapeptide lactone rings are bound via amide bridges to the phenoxazone chromophore, which is responsible



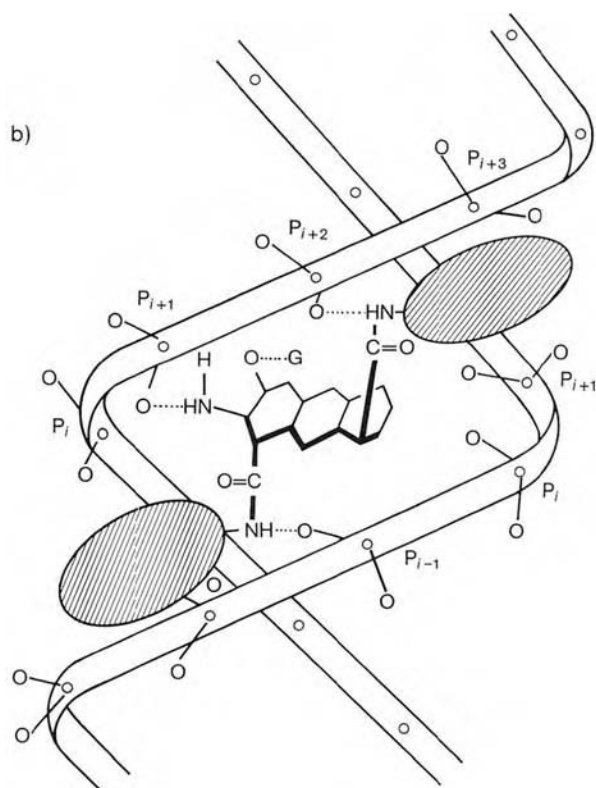
a)



**Figure 2** Actinomycin D (=C1): a, Structure: Both peptide lactone ring systems are identical. Amino acid residues: Sar, sarcosine; L-N-MeVal, L-N-methylvaline; L-Tr, L-threonine; D-Val, D-valine; L-Pro, L-proline. b, Model of the actinomycin DNA complex: P<sub>i</sub> and P<sub>j</sub>, consecutive phosphate groups of both DNA strands. The actinomycin D molecule fills the small furrow of the double helix. DNA, deoxyribonucleic acid. (From Ref. 4.)

for the orange-red color. Specific interaction with the double-stranded DNA results from the insertion of the phenoxazine ring system between two (GC) base pairs of the double helix. A further interaction results from the specific hydrogen bridge between the deoxyguanosine of the adjacent strands and the two peptide rings, which lie in the little furrow of the DNA (1) (Fig. 2b), rendering the DNA unusable for the RNA polymerase. Transcription but not translation (2), is inhibited. In vivo the antibiotic binds preferentially to the active chromatin regions (3). Obviously actinomycin D hardly impairs DNA synthesis.

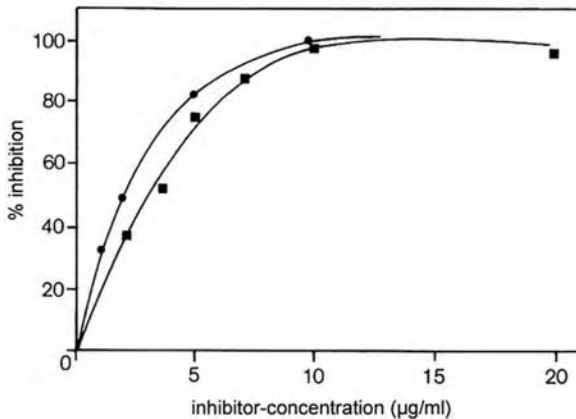
A representative example is the application of actinomycin D for the analysis of the light-controlled increase of phenylalanine ammonia lyase activity. By using cell suspension cultures of parsley (4) it was shown that the light-dependent increase is inhibited by actinomycin D (Fig. 3). This first hint of RNA synthesis as a step in the signal chain between light induction and enzyme increase was finally confirmed in cell free systems, in which light triggered the production of messenger RNA (mRNA) (6). Because transcription is followed by translation, the activity increase in cell cultures is necessarily blocked also by the translation inhibitor cycloheximide.



**Figure 2** Continued.

Phenylalanine ammonia lyase (PAL) occupies a key position in the biosynthesis of secondary compounds. Biosynthetic pathways branch off from the reaction product cinnamic acid to lignin, flavonoids, and stilbenes as well as coumarins and phenolic acids. Therefore it becomes evident that actinomycin D, for instance inhibits the light-induced anthocyan synthesis, if the drug is applied before light induction (7). Blocking PAL specifically by the amino oxy analog of phenylalanine, L- $\alpha$ -amino oxy- $\beta$ -phenyl propionic acid (AOPP), therefore, also inhibits the synthesis of the substance classes mentioned (8).

Paradoxical effects of actinomycin D that initiate increased enzyme activities or trigger morphogenetic processes (e.g., rooting of isolated cotyledons) do not necessarily oppose the effect of the antibiotic as a transcription inhibitor. It was shown for the wound-induced ribonuclease (RNase) of cabbage turnip slices that enzyme activity (superinduction) is increased by the antibiotic if the injury is set at least 45 min before



**Figure 3** Inhibition of light-induced phenylalanine ammonia lyase by actinomycin D (■) and cycloheximide (●) in cell suspension cultures of parsley. Antibiotics were added 30 min before light induction. Enzyme reaction started 7 h after light induction. (From Ref. 5.)

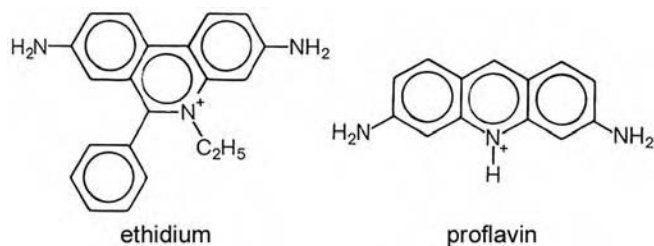
application of the antibiotic (9). This is intelligible if RNase production depends on two factors: a long-lasting mRNA for the enzyme and a short-lived mRNA for a repressor protein, which inhibits the translation of RNase-specific mRNA.

## 2. Ethidium and Acridines

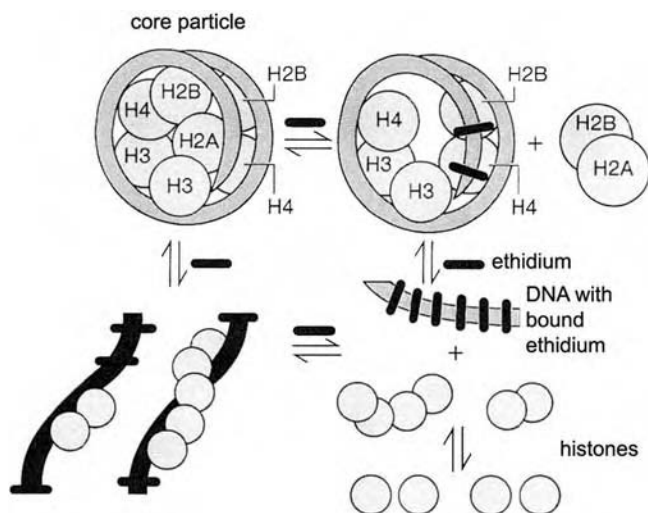
Ethidium and acridines such as proflavin are synthetic intercalating polycyclic aromates (Fig. 4). These dyes are known for their mutagenic and carcinogenic effects. Their application leads to conformational changes of the DNA double strand.

In eukaryotes, the nonspecific intercalators bind primarily at the end of the DNA termini of nucleosome core particles (10). Initially, this process leads to the release of one H2A and one H2B histone, exposing new DNA binding sites. Further ethidium binding results in a complete dissociation of the nucleosome particle to liberate DNA and histones. The ethidium-induced dissociation is reversible. Figure 5 shows the schematic course.

Ethidium-related conformational changes not only reduce the stability of the histone DNA complex, which indirectly can disturb gene expression, but also directly impair RNA and DNA polymerases and alter topoisomerase effects (see later discussion).



**Figure 4** Ethidium and proflavin.



**Figure 5** Model for the binding of ethidium to chromatin on the level of nucleosome core particles and for ethidium-induced dissociation of histones. DNA, deoxyribonucleic acid. (From Ref. 10.)

An ethidium or acridine treatment causes strand breaks in circular DNA, which subsequently impair DNA replication and transcription. Acridines and ethidium are also used to obtain a depletion of mitochondrial DNA in order to generate respiration-deficient mutants in yeast cells (11). The DNA losses can also be triggered in chloroplasts (12).

### 3. Caffeine

The trimethylxanthin caffeine (formula cf. Fig. 72) also belongs to the DNA intercalating substances. Since it primarily binds to

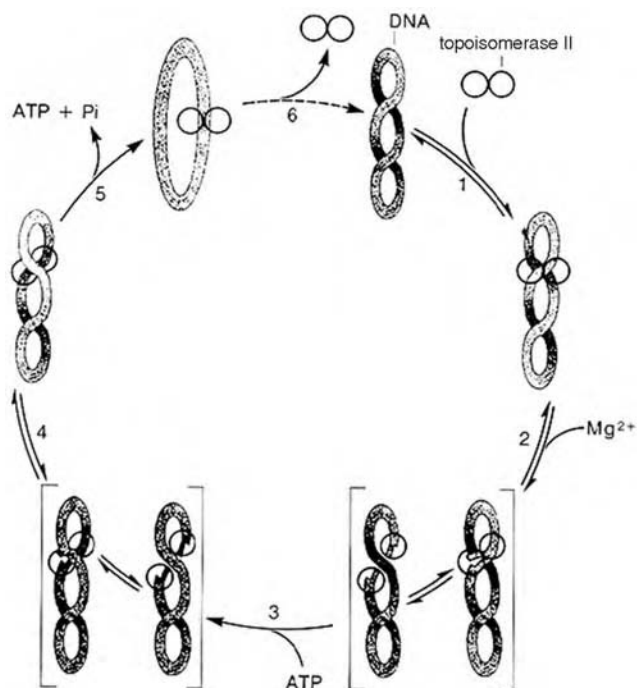
damaged DNA regions, DNA repair and the photoreactivation, which remove ultraviolet (UV)-induced damage, are inhibited. Thus caffeine strengthens mutagenic and lethal effects of genotoxic substances. Its effect on microtubules (cf. below) has to be distinguished from this activity.

### C. Topoisomerase

The genetic code is determined by the one-dimensional sequence of nucleotides. In addition, the topological properties of DNA determine the way genetic information is expressed and passed on to the next generation. As an example, three-dimensional changes such as supercoiling determine the initiation and elongation rates for DNA replication and transcription. Topological problems also have to be solved during recombination as well as the separation of the daughter chromatids during cell division.

Topoisomerases I and II are responsible for the alteration of the DNA topological characteristics by a change of the linking number, the number of turns made by one strand about the other. At first the function is explained for the topoisomerase II. This enzyme resembles the bacterial gyrase, which for obvious reasons is also found in chloroplasts and mitochondria. Topoisomerase II changes the DNA topological features by breaking and rejoining double-stranded DNA. As shown in Fig. 6 for a circular DNA found in chloroplasts and mitochondria, topoisomerase II cuts both strands, passes another portion of the double-stranded DNA through the cut, and reseals the cut in an adenosine triphosphate-(ATP)-dependent process. This model shows the catalytic cycle for the elimination of negative superhelical twists. Topoisomerase II is important as well for the topological characteristics of chromosomes of the cell nucleus. During the interphase chromatin is organized in long loops, associated with a flexible protein scaffold, and anchored at the nuclear membrane. The transition to mitosis or meiosis coincides with coiling of the scaffold into a helix and further packing. This condensation process yields the transport form of chromosomes. Decondensation again takes place at the transition to the next interphase. The eukaryotic topoisomerase II can relax both positive superhelices (with an excess of supercoils) and negative superhelices (with a diminished number of supercoils). Topoisomerase I merely carries out a single strand break. The relative rotation of the two separate DNA ends and the following ligation lead to the relaxation of the DNA, i.e., the removal of supercoils.

A survey of the most important inhibitors is found at Liu (14).

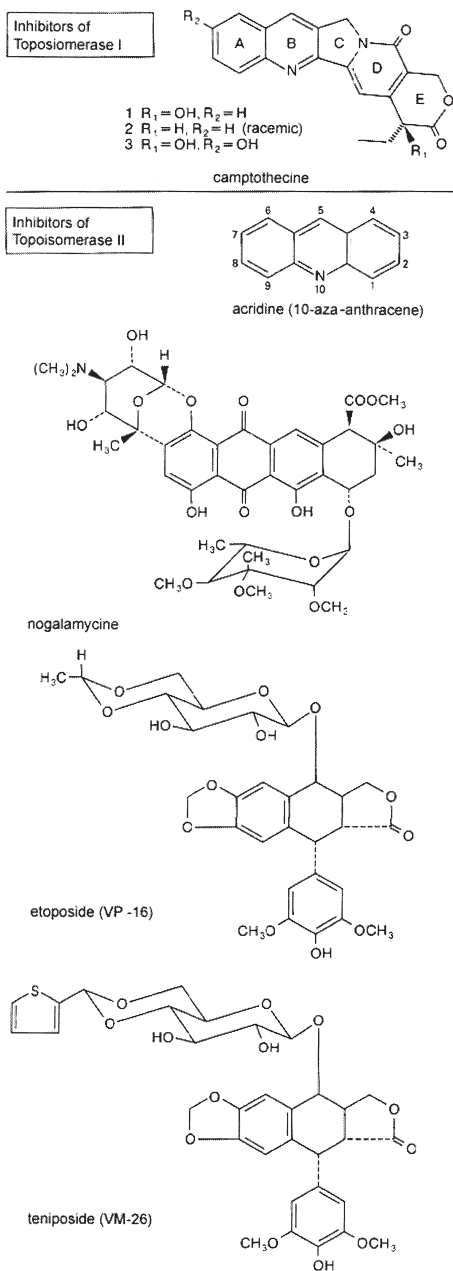


**Figure 6** Catalytic cycle of topoisomerase II. ATP, adenosine triphosphate; DNA, deoxyribonucleic acid;  $P_i$ , (From Ref. 13.) The monodimer topoisomerase II binds to DNA (1), cuts both DNA strands (2), passes another portion of the double-stranded DNA through the cut (3), reseals the cut in an ATP-dependent process (4), and is released after ATP-hydrolysis (5).

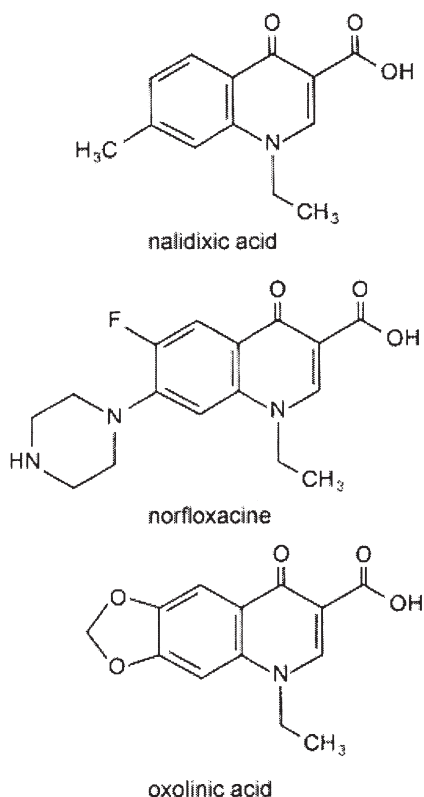
### III. INHIBITORS OF THE DNA TOPOISOMERASE II AND I

In principle the topoisomerases of plants can be inhibited by the same inhibitors as the respective mammalian enzymes in which applications are focusing on chemotherapy (antitumor effects). These drugs include intercalating dyes from the group of acridines and anthracyclins (Fig. 7a) as well as actinomycin D (Fig. 2). Under the influence of these inhibitors topoisomerase II-induced strand breaks occur, lacking subsequent religation.

The nonintercalating epipodophyll toxins VP 16 (etoposide; Fig. 7a) and VM 26 (teniposide) are glycoside derivatives of the podophyll toxins, inhibitors of tubulin polymerization. For VM 26 the existence of topoisomerase II was proved in nuclear extracts of corn embryos (15). For this purpose the plasmid pBR 322 was transformed from the superhelix



**Figure 7** Inhibitors: a, of eukaryotic topoisomerases I and II; b, of prokaryotic topoisomerase II (= gyrase).



**Figure 7** Continued.

into the linear form in the presence of the enzyme, the inhibitor, and ATP. The herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) stimulated *in vivo* the activity of the topoisomerase II twice, whereas abscisic acid (ABA) reduced the activity significantly. Large quantities of the enzyme were associated in yeast with chromatin (16).

Camptothecin (Fig. 7a) is one of the inhibitors of topoisomerase I. It is a cytotoxic alkaloid found in the Chinese tree *Camptotheca acuminata* (Nyssaceae). Camptothecin primarily kills cells in the S phase. The toxin causes exchange of chromatids and chromosomal aberrations by single strand breaks in the presence of topoisomerase I. The enzyme remains covalently bound to the 3'-phosphorylated termini of the fractured DNA strands. Camptothecin binds to the DNA topoisomerase I complex; however, it does not bind the free enzyme or free DNA.



Another inhibitor is berenil, 1,3-tris(4'-amidinophenyl)triazine. This compound as well as camptothecin have proved to be effective inhibitors of topoisomerase I from tobacco cells (17).

The prokaryotic topoisomerase II (= gyrase) is also found in chloroplasts and mitochondria. It can be inhibited by the synthetic compound nalidixic acid and other chinolin compounds such as norfloxacin and oxolinic acid (cf. Fig. 7b). These inhibitors prevent the repair of the DNA double-strand breaks by blocking a covalent gyrase-DNA intermediate. The consequences among other effects, are inhibition of DNA replication and induction of DNA disintegration.

Prokaryotic gyrases, in contrast to eukaryotic topoisomerases, also induce the formation of positive and negative superhelices (supercoiling) from the relaxed DNA form in the presence of DNA.

The transformation of the relaxed DNA ring into the negative superhelix is a prerequisite for the replication of the genome of organelles and prokaryotes. The application of nalidixic acid causes the gradual loss of the organelle genome. The DNA rings disappear in the chloroplasts within two to three generations. The chlorophyll content is reduced. However, the replication of organelles is not affected (18).

#### IV. INHIBITION OF ENZYMES INVOLVED IN NUCLEIC ACID SYNTHESIS

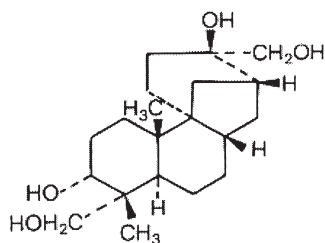
##### A. Aphidicoline

Deoxyribonucleic acid polymerase  $\alpha$  plays a most important role in the replication of chromosomal DNA in eukaryotes. It is directly and specifically inhibited by aphidicoline. This drug is a tetracyclic diterpene with four hydroxyl groups (Fig. 8) from the deuteromycete *Cephalosporium aphidicola*. The nuclear DNA polymerase  $\beta$ , a repair enzyme, and the mitochondrial DNA polymerase  $\gamma$ , a replication enzyme, are not inhibited (19).

Aphidicoline binds to the DNA polymerase complex, which is disabled by the binding process. It is assumed that a natural substance with a structural similarity to this antibiotic (e.g., steroid or another diterpen) regulates the cellular DNA replication about the same receptor site. Aphidicoline is used for synchronizing cells in the  $G_1 \rightarrow S$  transition phase.

##### B. $\alpha$ -Amanitin

$\alpha$ -Amanitin, a very toxic compound, is one of the main poisons of the deadly poisonous death cap (*Amanita phalloides*). The bicyclic octapeptide



**Figure 8** Aphidicoline.

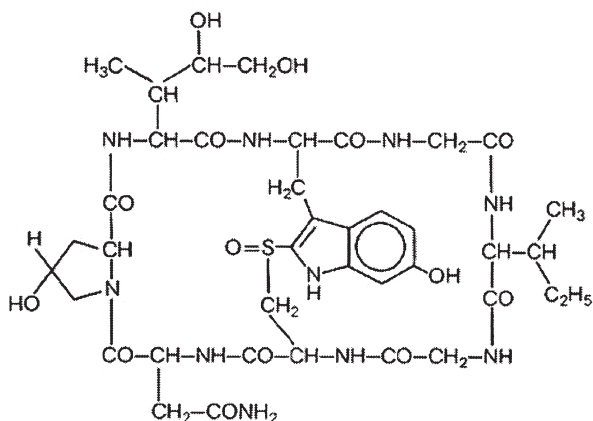
is cross-linked by hydroxy tryptathionine sulfoxide (Fig. 9). It specifically inhibits the DNA-dependent RNA polymerase II in the cell nucleus, which is needed for the synthesis of mRNA precursors. Other polymerases are not inhibited. Already by 1971, Seitz and Seitz (20) showed the selective inhibition of the synthesis of an adenosine monophosphate-(AMP)-rich RNA in cell suspension cultures of parsley.

## V. INTERFERENCE WITH THE METABOLISM OF NUCLEOTIDES

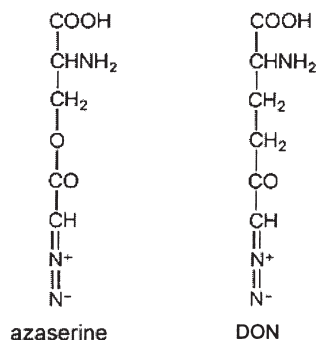
A variety of antibiotics and similar compounds are available to interfere with nucleic acid functions at the level of the nucleotide metabolism. The most important effects can be divided into three groups:

1. Inhibition of the purine and pyrimidine synthesis, e.g., by azaserine and deoxynivalenol (DON, Fig. 10) from streptomycetes. Because of their inhibition of purine synthesis they are known as antitumor antibiotics.
2. Inhibition of nucleotide synthesis. 5-Fluorouracil and 5-fluorodesoxyuridine (Fig. 11) are converted *in vivo* into 5-fluorodesoxyuridylate. They specifically inhibit the thymidylate synthase. The DNA synthesis is then impaired by the lack of thymine nucleotides.

In hypocotyls of mustard embryos inhibition of the DNA synthesis could already be observed 30 min after application of the inhibitor ( $4 \times 10^{-5}$  molar) (21). On the other hand, an inhibition of elongation growth was recorded only after c. 6 h. Both processes were restarted with lag phases comparable to previous lag phases by addition of thymine ( $4 \times 10^{-4}$  molar).



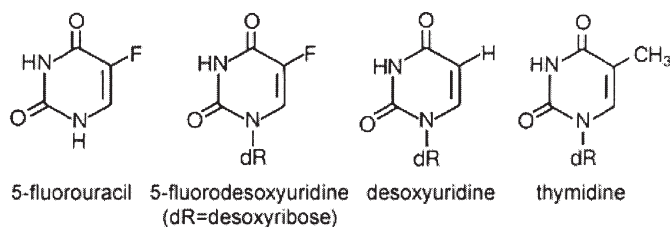
**Figure 9**  $\alpha$ -Amanitin.



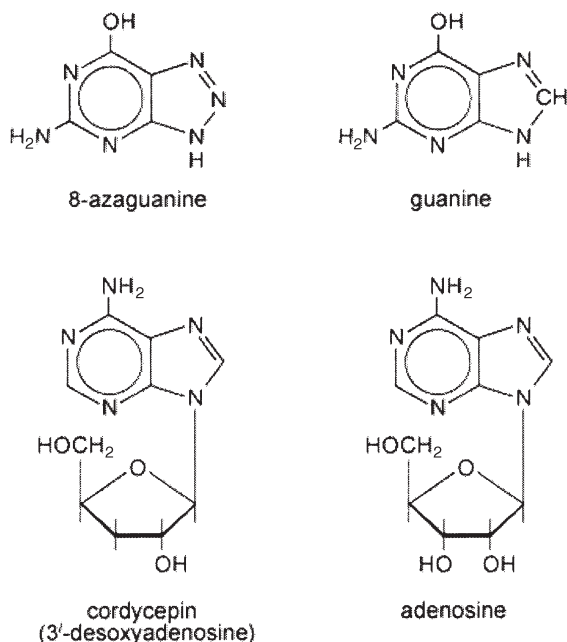
**Figure 10** Inhibitors of purine and pyrimidine synthesis. DON, deoxynivalenol.

5-Fluorouracil inhibits a number of morphogenetic processes. An example is fruit body formation of the ascomycete *Sordaria fimicola*, which is already selectively inhibited at 1- $\mu$ M concentrations without impairing vegetative growth (22).

Morphogenetic effects were also observed in *Zinnia elegans* (23). 5-Fluorodesoxyuridine (FdU) prevents the differentiation of vessels in isolated mesophyll cells, which are frequently formed after a cultivation time of 48 h. Comparable inhibitory effects were also noticed in other systems. It is assumed that this effect cannot be explained exclusively by the inhibition of the thymidylate synthase (after transformation of FdU to flourodesoxyuridine monophosphate), but may also depend on the integration of FdU into DNA.



**Figure 11** Nucleotide synthesis inhibitors compared to thymidine.



**Figure 12** 8-Azaguanine, guanine, cordycepin, and adenosine.

- Integration of nucleotide analogs into the RNA and DNA: a number of natural and synthetic analogs of nucleic acid components can be converted to nucleotide analogs. After integration into the nucleic acids the properties of the macromolecules are altered in different ways. An important example is the synthetic compound 8-azaguanine (Fig. 12). This guanine analog is changed to the corresponding adenosine analog and integrated into the RNA, followed by disturbances of the codon–anticodon recognition in the protein synthesis (discussed later). The nucleoside analog cordycepin (3'-desoxy

adenosine, Fig. 12) is also frequently used. It is attached to the 3' end of growing RNA chains. It blocks further prolongation. The formation of the poly(A)-segment at the 3' end of the mRNA precursors in the cell nucleus and therefore the production of mRNA are particularly inhibited. The antibiotic, which was initially isolated in 1951 from the ascomycetes *Cordyceps militaris* and later also from *Aspergillus nidulans*, is now produced synthetically. Cordycepin was used for measuring the stability of mRNA in plants (24).

## VI. INTERFERENCE WITH RIBOSOMAL FUNCTIONS

### A. Basics

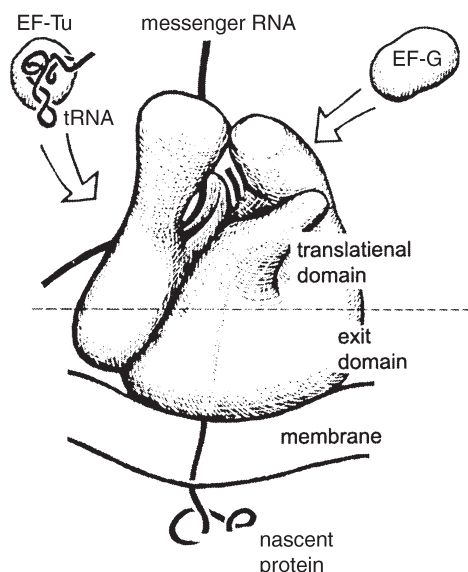
During the second stage of the information transfer from gene to protein (translation) the nucleotide sequence of the mRNA is translated into a chain of amino acids, which form the polypeptide chain. Many antibiotics interfere with this process.

The ribosome plays a crucial role in protein synthesis (Fig. 13). It is involved in the translation of the mRNA for the production of the proteins. Significant size differences exist between the cytosolic ribosomes of the eukaryotes on the one hand and the organelles as well as the prokaryotic ribosomes on the other hand, represented by different S values. The first group consists of 80S ribosomes, the second of 70S ribosomes. The exact values can vary somewhat among different organisms. Each ribosome is built up from two subunits of different size: The 80S ribosomes consist of 40S and 60S subunits, the 70S ribosomes of 30S and 50S subunits. The composition of ribosomal ribonucleic acid (rRNA) and proteins and the topographical location of the individual components are known.

### B. Ribotoxins

A considerable number of plants produce ribosome inactivating proteins (RIPs), which have been extensively reviewed by Nielsen and Boston (26). As a biological function, protection from virus infections by ribosome inactivation is assumed. Reports in 1997 linked the expression of some RIP genes to various stresses and even circadian control (27).

The RIPs are classified into three groups based on their physical properties (28). Type I RIPs, such as pokeweed antiviral protein (PAP), saporin (from soapwort, *Saponaria officinalis* L.), and barley (*Hordeum vulgare*) translation inhibitor, are monomeric enzymes, each with an approximate molecular weight (MW) of 30,000. These basic proteins share a



**Figure 13** Model of a (membrane-bound) ribosome with the binding sites of messenger ribonucleic acid (mRNA) as well as different prokaryotic elongation factors. The nascent polypeptide chain leaves the ribosome in unfolded conformation. tRNA, transfer ribonucleic acid; EF-Tu, elongation factor Tu; EF-G, elongation factor G. (From Ref. 25.)

number of highly conserved active site residues and secondary structures within the active site region but are distinctly different in overall sequence homology and posttranslational modifications (29). To date, most RIPs that have been characterized fall into the type I class (30).

Type II RIPs, such as ricin and abrin, are highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of an approximate MW of 30,000 (31). One polypeptide with RIP activity (A chain) is linked to a galactose binding lectin (B chain) through a disulfide bond (32). The lectin chain can bind to galactosyl moieties of glycoproteins and/or glycolipids found on the surface of eukaryotic cells and mediate retrograde transport of the A chain to the cytosol. Once it reaches the cytosol, the RIP has access to the translational machinery and readily disrupts protein synthesis.

Type III RIPs are synthesized as inactive precursors (proRIPs) that require that proteolytic processing events occur between amino acids involved in formation of the active site (28). These processing events result in proteins with tightly associated polypeptide subunits (33). To date, type

III RIPs have been characterized only from maize and barley. The two RIPs that have been characterized in maize are both type III proteins (34). Barley, in contrast, has one type I RIP and one type III RIP.

Some of these RIPs are among the most toxic compounds. One molecule per cell is sufficient to turn off its protein synthesis.

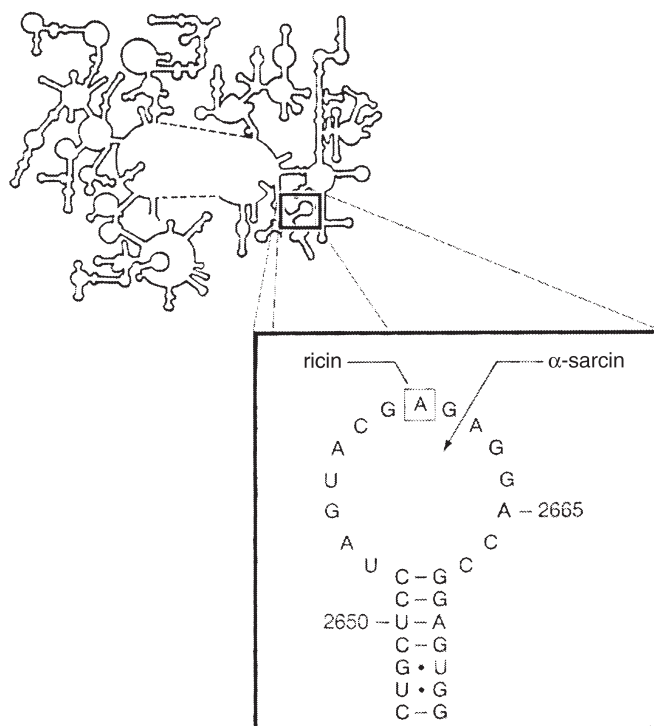
The site-specific *N*-glycosidase activity has long been the crucial property of RIPs. However, additional activities have also been reported. The most widespread of these is the activity of a polynucleotide, adenosine glycosidase, which depurinates DNA as well as RNA from sites other than the A 4324 of rRNA (35).

The RIPs differ in their requirement for cofactors, which contribute to their translational inhibitory activity. Gelonin from *Gelonium multiflorum*, barley RIP, pokeweed antiviral protein, and tritin-S from wheat require ATP for maximal activity, but other RIPs such as bryodin-R from *Bryonia dioica*, momordin from *Momordica charantia*, and saporin do not (36).

Type II RIP ricin from the Euphorbiaceae *Ricinus communis* has been examined in detail. It consists of an A and a B chain, which are held together by disulfide bridges. The B chain contains two galactose binding sites. The toxin can bind to a variety of cells and then cross the cell membrane. The A chain is a RNA *N*-glycosidase. It catalyses the removal of adenine from position A 4324 of the 28S rRNA (Fig. 14). This adenine lies within a 14-nucleotide region known as the  $\alpha$ -sarcin loop and is conserved in large rRNAs from bacteria to humans (38). A GAGA sequence in which the first A is the RIP substrate forms the core of a putative tetra-loop surrounded by a short base-paired stem (39). Irreversible modification of the target A residue blocks elongation factor (EF)-1- and EF-2-dependent guanosine triphosphatase (GTPase) activities and prevents the ribosome from binding EF-2, thereby interrupting chain elongation and blocking translation (40). The compartmentation of the toxin in the vacuole of endosperm cells protects the cytoplasmic protein synthesis in *Ricinus* spp. during seed germination (41).

A comparable mechanism of action is known for the type II RIPs abrin from *Abrus precatorius* (Leguminosae) and modeccin from *Modecca digitata* (*Adenia digitata*, Passifloraceae). These glycoproteins block protein synthesis in intact and cell free systems. The shiga toxin from the bacterium *Shigella dysenteriae* and the shiga similar toxin II version (SLT-II V) from *Escherichia coli* are also included.

Highly effective in plants but also in prokaryotes is the Mirabilis antiviral protein (MAP) from *Mirabilis jalapa* (Nyctaginaceae). It is a nonglycosylated type I RIP, which shows a considerable homology to the ricin A chain. In addition, MAP causes removal of adenine. The RIP petroglauin from *Petrocoptis glaucifolia* (Caryophyllaceae) that appears



**Figure 14** Target sites of ricin and  $\alpha$ -sarcin using the 23S ribosomal ribonucleic acid (rRNA) of *Escherichia coli* as an example. (From Ref. 37.)

endemically in the north of Spain has comparable activities. For instance, it inhibits protein synthesis in extracts of *Vicia sativa* and to a lower extent the wheat germ system (42). The antiviral protein phytolaccin from roots of *Phytolacca americana* (Phytolaccaceae) shows structural similarities to the A chains of ricin, abrin, and modeccin, as do gelonin from seed of *Gelonium multiflorum* (Euphorbiaceae), crotin from seeds of *Croton tiglium* (Euphorbiaceae), as well as curcin from seeds of *Satropa curcas* (Euphorbiaceae).

An overview of type I RIPs is given by Merino and associates (43). Particularly high inhibitor concentrations appear in *Lychnis flos-cuculi*, *Euphorbia serrata*, and *Capsella bursa-pastoris*.  $\alpha$ -Sarcin, which is secreted by *Aspergillus giganteus*, shows a significant homology to the *Aspergillus restrictus* toxins mitogillin and restrictocin. At first the type I RIP  $\alpha$ -sarcin was noticed for its antisarcoma effect. It is an RNase, which splits the phosphodiester bond on the 3' position of G 4325 (44) (cf. Fig. 14).



The inhibitory effect of this toxin was proved for wheat shoot extracts as well as yeast. However, entry into the living cell is restricted.

Although the enzymatic mechanism of RIP activity is well defined, the physiological steps leading to cell death are not well understood. The RIP-treated cells showed the morphological features characteristic of apoptosis, including condensation and fragmentation of cell nuclei, cytoplasmic densification, breakdown of nuclear DNA into discrete fragments, and mitochondrial membrane alterations (45, 46).

Ectopic expression of PAP and the type III barley RIP JIP60 produced abnormal phenotypes in transgenic tobacco. For PAP, only those plants providing large amounts of enzymatically active proteins produced morphologically aberrant plants (47).

The most common bioassays that determine RIP antifungal activity are based on the inhibition of fungal colony growth (48, 49). However, microscopic examination of fungi has offered some insight into the potential effect of RIP treatment on individual fungal cells. Hyphae of *Trichoderma reesei* treated with two type I RIPs from *Mirabilis expansa* were found to be narrower than control hyphae and to have extensive septum formation and enlarged tips (50). Treatment of *Aspergillus nidulans* spores with an active form of maize RIP led to normal germination, but autolysis of hyphae occurred before septum formation. Autolysis depended on both ribosome-inactivating activity of the protein and G-protein signaling in the cells (51). Confocal imaging of maize RIP showed localization of RIP within the cell and a decrease in nuclear staining before the autolytic event (K Nielsen, GA Payne, RS Boston, unpublished results). These observations suggest that fungi, as do mammalian cells, have a programmed cell death pathway. Nuclear degradation observed before autolysis in *A. nidulans* cells is analogous to the programmed cell death observed with RIP treatment of mammalian cells. Taken together, the observations discussed suggest that the effect of RIPs on individual cells from various organisms may be manifested by similar pathways.

## VII. PROTEIN SYNTHESIS

Protein synthesis at the ribosomes consists of three different functional steps: (a) initiation, which launches the synthesis; (b) elongation, a cyclic process in which the available peptide chain is extended by an amino acid; (c) termination, which stops protein synthesis. Each individual step depends on a number of factors, i.e., proteins that are temporarily bound to the ribosome. The complete process is energy-dependent. A selection of inhibitors of the protein synthesis is summarized in Table 1.

**Table 1** Inhibitors of Protein Synthesis

Compound	Target	Inhibition reaction <sup>a</sup>
(a) Initiation		
Aurintricarboxylic acid	80S and 70S Ribosomes	Inhibits mRNA binding at the small ribosomal subunit
Edeine A	80S and 70S Ribosomes	Blocks P site of the small ribosomal subunit
Streptomycin	70S Ribosomes of plastids and prokaryotes	Destabilizes initiation complex and reading errors at the translation of mRNA
Kasugamycin	70S Ribosomes of prokaryotes; protein synthesis of distinct fungi	Inhibits fMet-tRNA binding
(b) Elongation		
Tetracycline	80S and 70S Ribosomes	Inhibits adhesion of aminoacyl-tRNA at the A site of ribosomes
Puromycin	80S and 70S Ribosomes	Causes premature chain break
Cycloheximide	80S Ribosomes	Binds at the 60S subunit; inhibits translocation
Chloramphenicol	70S Ribosomes	Inhibits peptidyltransferase reaction
Erythromycin A	70S Ribosomes	Binds at the 50S subunit; inhibits translocation

<sup>a</sup>mRNA, messenger ribonucleic acid; fMet, formylated methionine; tRNA, transfer RNA.

Before their integration into the polypeptide chain, amino acids are activated by binding to specific transfer RNA (tRNA) molecules. Therefore, they reach the ribosome as aminoacyl-tRNAs. The specificity of the protein synthesis is guaranteed by base pairing between the anticodon of the amino acid-loaded tRNA and the complementary codon of the mRNA. This process takes place at the ribosome just as the linkage of peptide bonds.

Since protein synthesis in plant cells takes place (a) in the cytosol (eukaryotic type) and (b) in the plastids and mitochondria (prokaryotic type), different inhibitors are effective in knocking out protein synthesis in these compartments.

## VIII. INITIATION

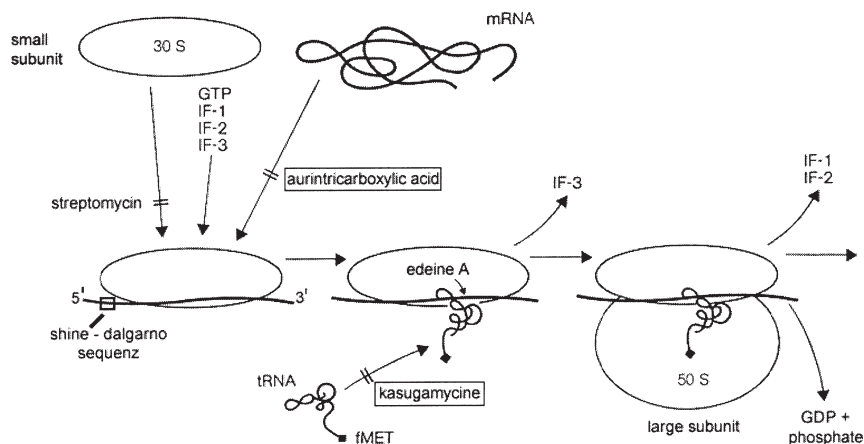
### A. Prokaryotes

In prokaryotes operons represent the transcription units. They consist of several genes each. In the case of protein-encoding genes, transcription of an operon leads to the production of a polycistronic mRNA encoding several polypeptides. Because there are multiple start sites for protein synthesis, different cistrons can be read independently during translation of a polycistronic matrix. The ribosome binds to the start point of a cistron and translates the nucleotide sequence into a polypeptide chain until the cistron terminus is reached; there the ribosomal subunits are released. Each cistron carries a marker, which is recognized as a starting point for the assembly of the translation complex. It is called the *Shine–Delgarno sequence* after its discoverers and is located near the start site for each of the encoded proteins, the initiator triplet AUG, which is complementary to the 16S-rRNA of the small ribosomal subunit.

Figure 15 further details the initiation as it is known for the prokaryotic as well as in principle for the mitochondrial and plastidial protein synthesis. In this case, the initiation tRNA carries a formylated methionine (fMet).

### B. Eukaryotes

Eukaryotic protein synthesis is more complex. Eukaryotic genes are usually physically separated in the DNA. Therefore, each gene is transcribed from its own start site, producing one mRNA, which usually yields a single protein. Since eukaryotic genes exist in pieces of coding sequences (exons), separated by non-protein-coding segments (introns),



**Figure 15** Initiation of the prokaryotic protein synthesis. The assembly of the translation complex involves the initiation factors (IF-1, IF-2, IF-3), GTP (as an energy source), and mRNA. This is followed by the binding of the initiator tRNA loaded with formylated methionine (fMet) (prokaryotic, mitochondrial, and plastidial systems) to the AUG start codon. This 30S initiation complex then binds the large subunit (50S). The target sites of several antibiotics are indicated. IF-1, interferon-1; GTP, guanosine triphosphate; mRNA, messenger ribonucleic acid; tRNA, transfer ribonucleic acid; fMet, formylated methionine. (Adapted from Ref. 52.)

the primary transcript must be processed to yield functional RNA. The eukaryotes are usually missing a Shine–Delgarno sequence. In eukaryotes the 5' cap (7-methylguanylate [ $m^7G$ ]) added during formation of the primary transcript determines the site of initiation. Figure 16 shows the most important steps (53). The  $m^7G$ -cap terminus is recognized by initiation factors. This set of proteins, called *eIF4*, includes a RNA helicase, which removes any secondary structure at the 5' end of the transcripts in an ATP-dependent reaction. Then the 40S ribosomal subunit binds as 43S preinitiation complex near the 5'-capped end of the mRNA. Then the subunit moves along at the matrix as on a rail. In this ATP-dependent reaction the nucleotide sequence is scanned until the initiating AUG triplet is found. Finally binding of the 60S subunit loaded with methionine transfer RNA (tRNA) completes formation of the 80S initiation complex.

Detailed descriptions of prokaryotic and eukaryotic protein synthesis are found in textbooks of biochemistry and cell biology, e.g., Lodish and colleagues (54).

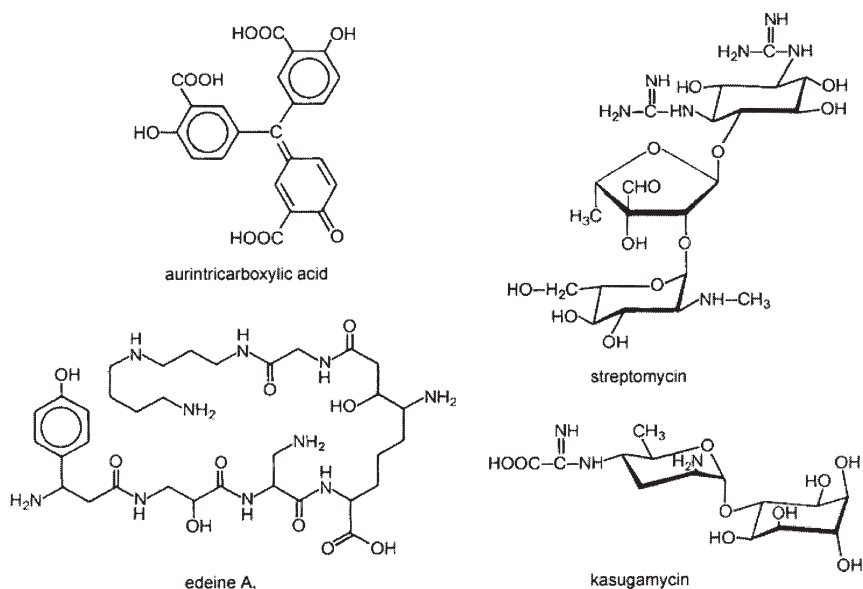


## IX. INHIBITION OF THE INITIATION

In a few cases the same inhibitor can interfere with both prokaryotic and eukaryotic initiation. Aurintricarboxylic acid, a synthetic dye in the group of the triphenylmethanes (Fig. 17), can be used in prokaryotic and eukaryotic cell free systems to block the initiation at distinct concentrations without impairing elongation and termination (*readout systems*). Aurintricarboxylic acid interferes with the binding of mRNA to the small ribosomal subunit. On the other hand, intact cells are hardly affected because of the poor uptake.

Edeine A, a basic peptide from *Bacillus brevis* (Fig. 17), shows similar effects. The antibiotic also binds to the small subunit, where it blocks the P site (see later discussion).

A number of aminoglycoside antibiotics that bind to the small ribosomal subunit exhibit a specific effect on the initiation phase of protein synthesis in prokaryotes and eukaryotic cell organelles. For this purpose, streptomycin is frequently used. It is an antibiotic from *Streptomyces griseus* (Fig. 17) that induces a conformational change of the ribosome, which in turn leads to the destabilization of the initiation



**Figure 17** Drugs and antibiotics that interfere with the initiation of protein synthesis.

complex. The consequences are reading errors. Furthermore, streptomycin also interferes with the elongation cycle (see later discussion).

Biogenesis of the photosynthesis apparatus of many algae, such as *Chlamydomonas*, *Scenedesmus*, or *Euglena* spp., can be suppressed by the specific inhibition of plastid protein synthesis, and pale forms are obtained. Crossing experiments by Sager and Ramanis (55) are known; in them the heredity of the plastid genes of *Chlamydomonas* sp. by crossing of the streptomycin-sensitive wild type with a streptomycin-dependent mutant was demonstrated.

Kasugamycin, another antibiotic of the aminoglycoside group (Fig. 17), is formed by *Streptomyces kanamyceticus*. Kasugamycin prevents the binding of fomylmethionine tRNA to the 30S subunit without causing reading errors. Most eukaryotic cells are insensitive. The fungus *Piricularia oryzae*, a rice pathogen, which can be eliminated by kasugamycin used as a pesticide, forms an exception. The mitochondrial protein synthesis is possibly affected in this case.

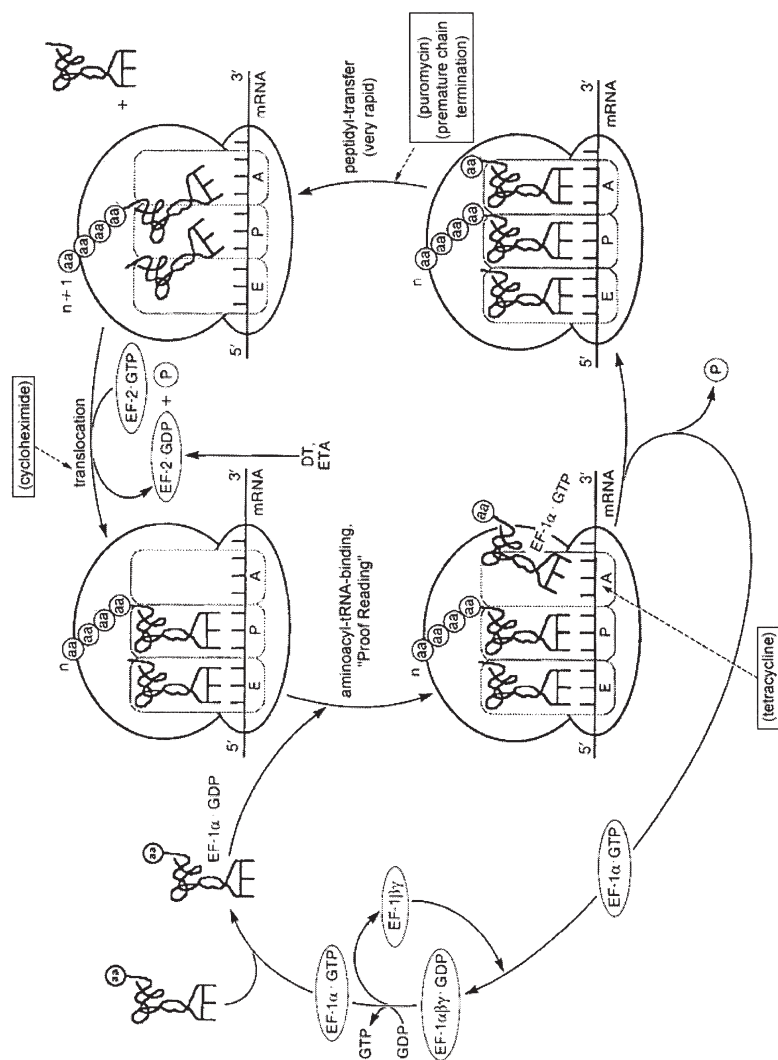
## X. ELONGATION CYCLE

The formation of the initiation complex is followed by a series of cyclic processes leading to the elongation of the peptide chain until the termination codon is reached. In both prokaryotic and eukaryotic protein synthesis, the ribosome–Met–tRNA complex begins the stepwise addition of amino acids by the in-frame translation of mRNA. Three different elongation factors, showing apparent homologies for the prokaryotic and eukaryotic components, are involved.

A scheme of the elongation cycle of eukaryotic protein synthesis (56) is provided by Fig. 18. Each ribosome has three codon-specific binding sites for tRNA molecules [three-site model (57)]: (a) a P(eptidyl) site for the tRNA, which carries the already synthesized peptidyl residues (or, after the initiation, the first amino acid residue), functions as donor site; (b) an A(cceptor) site, where a new aminoacyl tRNA binds codon-specifically as a ternary complex with the prolongation factor EF-1a (in prokaryotes, EF-Tu) and guanosine triphosphate (GTP); (c) an E(xit) site, which binds the deacylated tRNA. A spatial representation is given by Fig. 19.

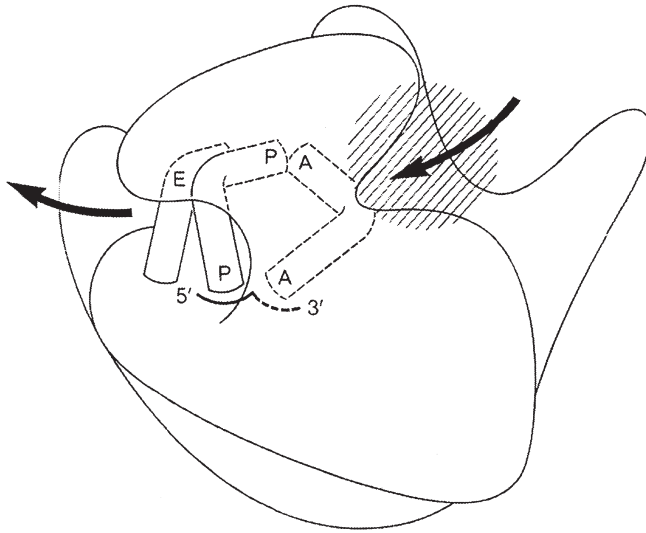
Chain prolongation proceeds in three steps (cf. Fig. 18):

1. An amino-acyl-tRNA binds to the A site on the ribosome and base-pairs with its corresponding codon in the mRNA.
2. A peptide bond is formed between the incoming amino acid and the growing chain at the P site, transferring the peptidyl



**Figure 18** Targets of several antibiotics that interfere with the eukaryotic elongation cycle. The antibiotics in brackets also inhibit prokaryotic protein synthesis. aa, Aminoacyl residue; mRNA, messenger ribonucleic acid; GDP, guanosine diphosphate; GTP, guanosine triphosphate; DT, diphtheria toxin; ETA, exotoxin A; EF, elongation factor. (Adapted from Ref. 56.)





**Figure 19** Spatial position of the A, P, and E sites for the ribosome-bound tRNAs. (From Ref. 58.) The arrows point to the suspected dissection of the tRNA during translation. The hatched area indicates the region of interaction with the elongation factor. The polarity of an mRNA segment is shown. mRNA, messenger ribonucleic acid; tRNA, transfer RNA.

chain to the incoming tRNA. The enzymatic activity for the peptidyltransferase reaction is provided by the 23S rRNA in the large ribosomal subunit. Peptide bond formation is accompanied by a movement of the incoming tRNA to the P site and of the now unloaded tRNA to the E site.

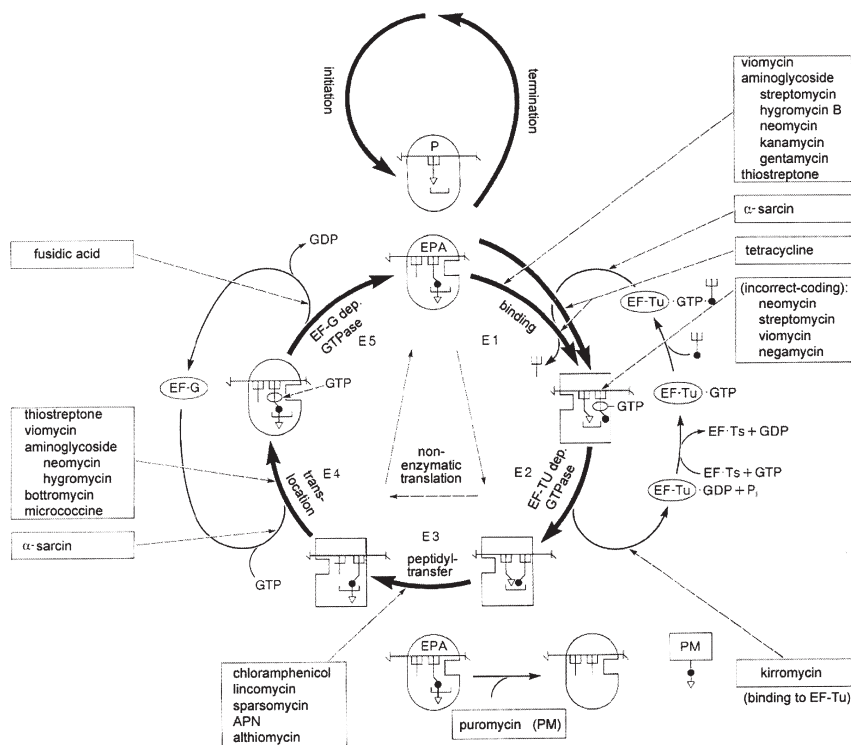
3. The ribosome translocates to the next codon, and the old tRNA is discharged from the ribosome.

The energy requirement is very high. Two GTPs per cycle are required for the synthesis of the aminoacyl-tRNA (aa-tRNA) at EF 1 and EF 2 as well as two ATP according to the equation



## XI. INHIBITION OF THE ELONGATION

Figure 18 shows the main targets for inhibitors of eukaryotic protein synthesis. The antibiotics highlighted in this scheme also block prokaryotic



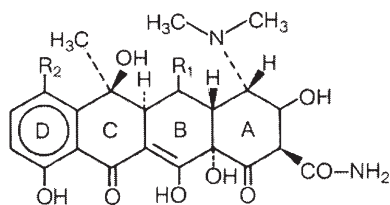
**Figure 20** Target sites of different antibiotics in the organelle-bound elongation cycle. GTP, guanosine triphosphate; GDP, guanosine diphosphate; APN, EPA, sites E, P, and A; EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts. (From Ref. 57.)

and organelle-bound protein synthesis. Selective inhibitors of the prokaryotic and organelle-bound protein synthesis are found in Fig. 20.

Tetracyclines (Fig. 21) specifically interfere with the binding of the aminoacyl-tRNA in lower concentrations to the acceptor site A in prokaryotes and eukaryotes. The basic structure of these antibiotics, which are produced by several *Streptomyces* species, consists of four linearly arranged six-carbon rings, the tetracycline system.

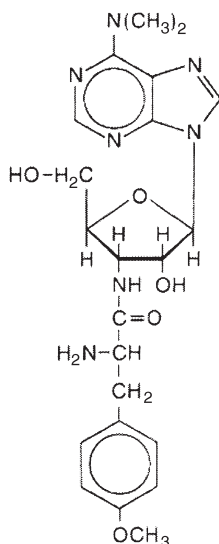
In addition to its function as an inhibitor of protein synthesis chlorotetracycline is known as a chelator for intracellular  $\text{Ca}^{2+}$  ions.  $\text{Ca}^{2+}$  ions become bound to membranes in the presence of the antibiotic, disturbing the dynamics of  $\text{Ca}^{2+}$  balance. This process results in heavy disturbances of growth, e.g., in the case of pollen tubes (59).

Several antibiotics inhibit the peptidyl transfer. Puromycin (Fig. 22) is a product of *Streptomyces alboniger*. Puromycin was the first antibiotic



tetracycline  $R_1=R_2=H$   
 chlortetracycline  $R_1=H$   $R_2=Cl$   
 oxytetracycline  $R_1=OH$   $R_2=H$

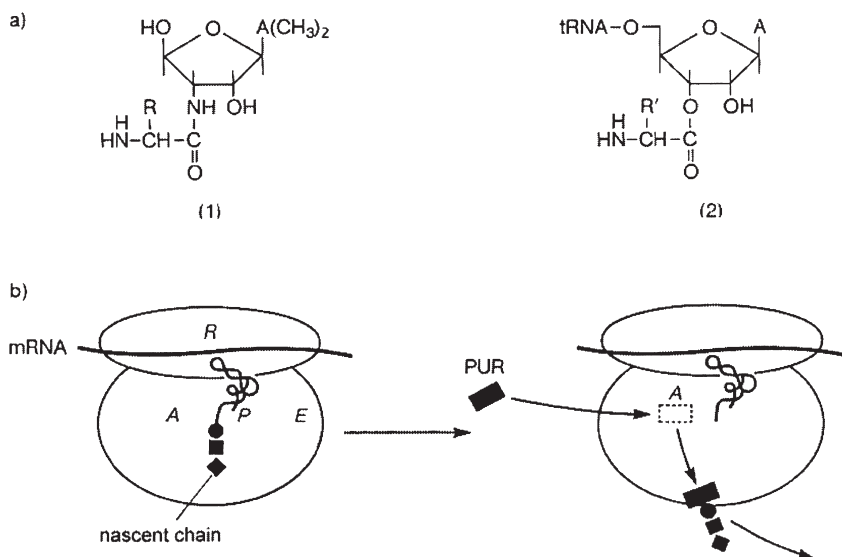
**Figure 21** Tetracycline antibiotics.



**Figure 22** Puromycin.

whose action mechanism could be elucidated on the molecular level. It is an analog of the aminoacyl adenosine terminus of an aminoacyl-tRNA (Fig. 23a). The aminoacyl residue is linked in the antibiotic by an amide bridge, but in the aminoacyl-tRNA by an ester bridge to the sugar residue. Puromycin binds to the A site and accepts the peptidyl residue of the growing polypeptide chain. Since the amide bridge of the antibiotic cannot be split by the peptidyl transferase, chain termination takes place (Fig. 23b). This process occurs in 80S and 70S ribosomes.

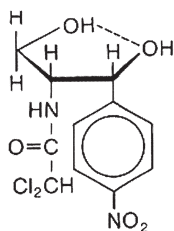
The peptidyl residue can only be transferred to puromycin if the peptidyl-tRNA is in the P site. On the other hand, no transfer can occur if



**Figure 23** Structure and action mechanism of puromycins: a, Puromycin (1) compared with the 3' end of an aminoacyl-tRNA (2). A, adenine; R, methoxyphenyl residue. R' side chain at the  $\alpha$ -C atom of the amino acid. tRNA, transfer ribonucleic acid. (From Ref. 60.) b, Action mechanism: At the beginning of the elongation cycle a peptidyl-tRNA occupies the P site. After binding of puromycin to the A site the antibiotic accepts the nascent polypeptide chain and detaches from the ribosome. (From Ref. 52.)

the peptidyl-tRNA occupies the site. This means that a positive puromycin reaction indicates a P site occupancy by the peptidyl-tRNA, a negative reaction to an A site occupancy (57).

A specific inhibition of the peptidyl transferase reaction in 70S ribosomes is achieved by chloroamphenicol (Fig. 24). Initially this antibiotic was derived from *Streptomyces venezuelae*. However, it was produced later synthetically. This simple compound is based on a phenylpropylamine structure. Only the D-threo isomer is effective. This characteristic can be useful for investigations of the specificity of the antibiotic effect. Chloroamphenicol binds to the large subunit of 70S ribosomes at the A site of the peptidyl transferase center. It interferes with the correct orientation of the aminoacyl residue of an aminoacyl-tRNA without impairing the binding to the mRNA. This prevents the recognition of the acceptor substrate by the peptidyl transferase. In addition, chloroamphenicol blocks the puromycin reaction. Chloroamphenicol causes the stabilization of the polysomes in addition to the inhibition of



**Figure 24** Chloroamphenicol (alicyclic ring form).

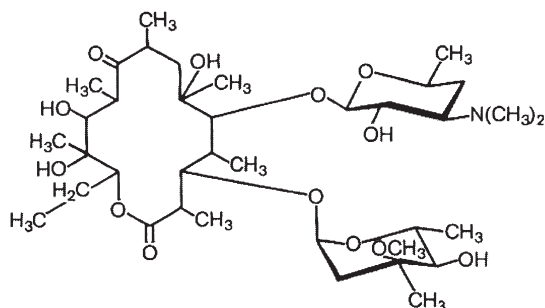
protein synthesis. This effect results in higher yields during isolation of membrane-bound polysomes.

Chloroamphenicol is also employed if the role of the nuclear and the chloroplast genome for the biogenesis of chloroplasts is to be determined. In certain species, e.g., pea, the *in vivo* results based on the differential inhibition of the plastid protein synthesis are consistent with *in vitro* approaches (55).

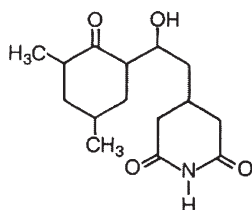
In addition to growth inhibition and strong reduction of cytochrome oxydase activity, chloroamphenicol increases the cyanide-resistant respiration (alternative oxidase) in suspension cultures of green soybean cells (61). The use of this antibiotic also indicates that side effects must be taken into account. The first report on the influence of chloroamphenicol on the plant deals with the inhibition of the ion uptake (62).

A representative of the macrolid antibiotics is erythromycin A. It is produced by *Streptomyces erythreus* and also interferes with 70S ribosomes. Its lacton ring carries two sugar residues (Fig. 25). The specific effect is based on the binding to the large ribosomal subunit. Through this the correct orientation of the peptidyl residue of the peptidyl-tRNA as well as the binding of this structure to the ribosome are prevented. The consequence is an inhibition of the peptidyl transfer and translocation that results in the termination of protein synthesis. Chloroplast protein synthesis is specifically inhibited by erythromycin in tissue cultures of *Nicotiana sylvestris*, whereas no influence is observed for mitochondria (63).

Cycloheximide (=actidion), a glutarimide antibiotic with a cyclic ketone (Fig. 26), is produced by *Streptomyces griseus*. It selectively inhibits protein synthesis at 80S ribosomes. Therefore, neither mitochondrial nor plastid protein synthesis is affected, as a result of the specific binding to the large subunit of 80S ribosomes. Cycloheximide stops the translocation of peptidyl-tRNA from the A to the P site in the last step of the elongation cycle. The molecular basis for the observation that cycloheximide can also specifically block the initiation of the peptide



**Figure 25** Erythromycin A.



**Figure 26** Cycloheximide.

chain is unclear. The antibiotic improves stabilization of polysomes in the presence of puromycin.

De novo protein synthesis starts in early stages of seed germination. This event can be suppressed completely by cycloheximide. In spite of the highly specific effect of cycloheximide, which can easily be demonstrated in cell-free protein synthesis (64) (Table 2), possible side effects should not be disregarded if the antibiotic is applied to the intact system (whole plant, single organ). A quick and effective inhibition of the DNA synthesis is known for yeast, *Aspergillus* spp., and other organisms. A strong inhibition of the ion uptake of roots has been reported at concentrations of 1  $\mu\text{g/ml}$ , which possibly can be linked to a disturbance in the energy metabolism of higher plants (65). Cycloheximide effects on a variety of cellular functions were also found in the green alga *Chlamydomonas reinhardtii*. A distinction of primary and secondary effects, e.g., due to the time course appears, to be excluded (66).

There is a clear danger of misinterpretation if cycloheximide inhibition of, e.g., a morphogenesis step is taken as proof for the dependence of this process on protein synthesis. A molecular analysis as well as the use of alternative antibiotics are strongly recommended in this case.

**Table 2** The Effect of Cycloheximide and Chloramphenicol on Cell-Free Protein Synthesis in Wheat-Germ System<sup>a</sup>

Preparation	Radioactivity (cpm)
Watermelon mRNA <sup>a</sup>	180 740
Control without mRNA	3822
Watermelon mRNA + cycloheximide (15 µg/ml)	6153
Watermelon-mRNA + D-threo chloramphenicol (400 µg/ml)	198 814

<sup>a</sup>Incorporation of <sup>35</sup>S-methionine was measured in acid-precipitable polypeptides after 90 min. mRNA, messenger ribonucleic acid.

Source: Ref. 64.

Cycloheximide was produced by the Upjohn Company as the active ingredient of a fungicide with the trade name Actidione, which is directed against leaf rot and rust diseases. The use of this drug is only restricted because of its phytotoxic effects. It was also applied for fruit thinning in orange, grapefruit, and olive cultures. A similar effect is obtained with the cycloheximide stereoisomer naramycin B (67).

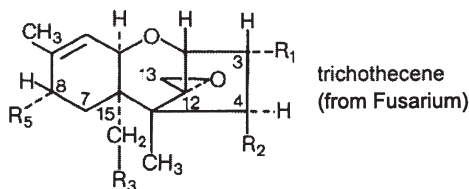
The aminoglycoside antibiotic streptomycin (Fig. 17) causes among others reading errors at the 70S ribosome. Obviously this effect is based on the binding to the 16S rRNA. For streptomycin-resistant mutants of *Nicotiana* spp. (68), one single nucleotide exchange (C replaces T) was reported for the 16S rRNA gene.

Several mycotoxins block the protein synthesis, too. Trichothecenes from *Fusarium* spp., e.g., T-2 toxin (Fig. 27), prevent chain elongation at 80S ribosomes. Trichothecenes such as T-2 cause fast inhibition of respiration in yeast in addition to inhibition of protein synthesis (69).

### A. Adenosine Diphosphate–Ribosylating Toxins

Chain elongation is among the most sensitive processes of protein synthesis. Ribosome modifications by plant and fungal toxins interfering with the normal interaction with the elongation factor EF-2 have been discussed before. Now covalent modifications of EF-2, which prevent an interaction with the ribosome, are discussed.

Comprehensive summaries have been published by Kato (70) and Perentthesis and coworkers (37). ADP-ribosylating toxins are the diphtheria toxin (DT) from *Corynebacterium diphtheria* and the *Pseudomonas* exotoxin A (ETA) from *Pseudomonas aeruginosa*, which were examined in detail because of their toxic effect on humans. Both consist of an A and a B subunit. The toxic enzyme activity is located in A, the binding ability to a cell membrane receptor, an anion antiporter, in B. The diphtheria toxin



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>5</sub>
diacetoxyscirpenol	OH	OAc	OAc	H
T-2 toxin	OH	OAc	OAc	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
neosolaniol	OH	OAc	OAc	OH
HT-2 toxin	OH	OH	OAc	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>

**Figure 27** Trichothecenes. T-2, trichothecene mycotoxin, T-2 toxin; HT-2, has a hydroxyl group substitution at R<sub>2</sub>. T, trichothecene; H, hydroxyl substituent.

catalyzes the inactivation of EF-2. This results from a transfer of ADP ribose from oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>) to the protein according to the following equation:



This reaction is practically irreversible and takes place at ETA in the same way.

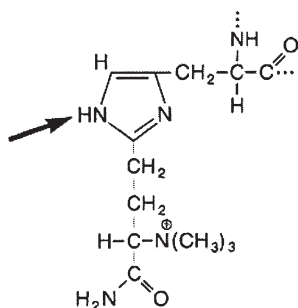
The recognition site on EF-2 is diphtamide (Fig. 28), an unusual amino acid that arises from the posttranslational modification of a histidine residue. This coincides with the recognition site for a Ca<sup>2+</sup>-dependent protein kinase III. It shows a similar specificity for EF-2 as DT and ETA. This kinase is activated by a multitude of hormonal stimuli. It would not be surprising if the well-known cytokine influence on ribosomes would also be carried out this way.

The effect of DT on plant cells was proved by the expression of the A chain in tobacco cells (71). A gene construct from plant promotor and DT gene was produced to provide a negative selection method for the functional test of plant promoters. The expression of the A chain proved to be toxic.

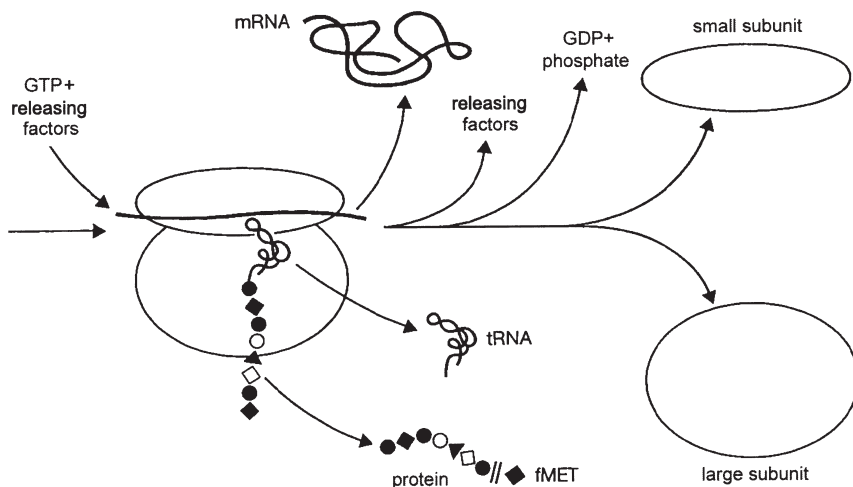
## B. Peptide Chain Termination

If a stop codon (UAA, UGA, or UAG) appears in the A site, then the peptide chain is complete. No tRNA has an anticodon that matches a stop codon. Whereas bacteria have three proteins with codon-specific termination recognition (release factors 1–3 [RF1–3]) whose shape is





**Figure 28** Structure of diphthamide. The arrow shows the ADP-ribosylating site. ADP, adenosine diphosphate. (From Ref. 37.)



**Figure 29** Chain termination and release of the involved components from the ribosome. mRNA, messenger ribonucleic acid; GTP, guanosine triphosphate; GDP, guanosine diphosphate; tRNA, transfer RNA; fMet, formylated methionine. (From Ref. 52.)

thought to be similar to that of tRNAs, eukaryotes have only two releasing factors (eRF1 and RF3). RF3 is a GTP-binding protein and acts in concert with the codon-recognizing factors to promote cleavage of the peptidyl-tRNA. This process releases the completed protein chain. This mechanism allows an efficient termination and reuse of the ribosomes and the mRNA for the next translation cycle. Figure 29 shows a scheme for the termination at prokaryotes.

Numerous antibiotics interfere with termination reactions, particularly compounds that inhibit the peptidyl transferase, e.g., chloroamphenicol and tetracycline. This finding is an indication that the formation of peptide bindings and the splitting of the ester bond between polypeptide and tRNA at the termination are carried out by a single enzyme.

## **XII. DISTURBANCE OF LIPID SYNTHESIS AND MEMBRANE FUNCTIONS**

Biomembranes form the outer border of the cell and allow intracellular compartmentation to segregate intracellular events from one another. The principal compounds are phospholipids and proteins, which are associated by noncovalent bonds. Phospholipids form a bimolecular film in which the polar head groups point to the outside, whereas the long hydrocarbon fatty acyl side chains in each leaflet are oriented toward one another, forming a hydrophobic core. Phospholipids serve as barriers for the passage of polar molecules. In contrast, proteins are responsible for most of the dynamic membrane processes. They function as receptors, pumps, channels, and transporters. Some of them have enzymatic activities. Integral and peripheral proteins can be distinguished (cf. Section 1). Membranes are 7–10 nm in diameter. They principally form delineate closed compartments. The asymmetrical construction of membranes causes differences between their outer and inner faces. Membrane components are to a certain extent laterally mobile within their membrane leaflet. However, they barely can rotate vertically around their axis.

According to the different constituents of membranes there are two scenarios for the membrane passage of compounds, which are based on two entirely different mechanisms:

1. *Free permeation or passive diffusion:* Small hydrophobic and small uncharged polar molecules can pass through the lipid bilayer according to their distribution coefficients. Phospholipid bilayers are slightly permeable to water; the main passage of water is facilitated by water channels, lined by aquaporins. Small increases in extracellular osmotic strength cause a rapid outflow of water from the cell, which is the basis for plasmolysis.

2. *Specific transport:* This kind of translocation is mediated by proteins. It is specific and generally faster than passive diffusion. Special translocators are available for this purpose: ATP-powered pumps (ATPases), ion channels, and transporters. Pumps use the energy released

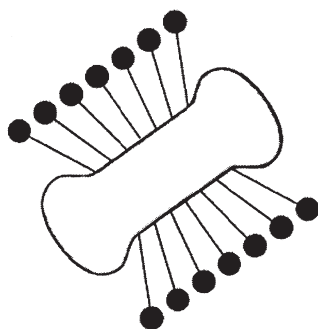
by ATP hydrolysis to drive specific ions such as  $H^+$  across the membrane against their electrochemical gradient. Channels catalyze the movement of water and ions (e.g.,  $K^+$ ) down their electrochemical gradient. Transporters facilitate the movement of ions or specific small molecules. Three mechanisms are distinguished for transporters: (a) unidirectional transport: uniporters transport a single type of molecule (e.g., amino acid) down its concentration gradient; (b) cotransport; (c) antiport. The term *active transport* is sometimes used to refer to uniporters and antiporters because they catalyze “uphill” movement of certain molecules.

An attack on membranes, which leads to the loss of the compartmentation, causes cell death. Membrane destruction frequently happens (a) by lipid peroxidation (cf. Section 4); (b) by tensides (detergents) and similar surface active compounds, which solve proteins from membrane by generating micelles (e.g., Triton X 100, digitonin, Na cholate); (c) by transport antibiotics, which often selectively permeabilize the membrane by formation of a pore structure; (d) by disturbance of specific transport, e.g., at the level of the transport ATPases and ion channels; (e) in addition, mechanical damage, e.g., frost damage, is of considerable importance. This happens when membranes are injured by ice crystal formation and is manifested during thawing. Membranes of protoplasts and organelles can also be destroyed by osmotic influences. They burst if osmotic water uptake beyond the elastic limit of the membrane takes place.

### A. General Disorganization of the Membrane

A number of surface-active compounds, e.g., tensides, lead to the destruction of the cell membrane. The effect is based on the solubilization of membrane proteins, which frequently involves the formation of tenside-protein micelles (Fig. 30). An extended part with hydrophobic properties and a polar head are characteristic of many tenside molecules. Figure 31 shows relevant examples. During micelle formation the hydrophobic domains point to the membrane protein, the polar groups to the aqueous phase.

Further surface-active compounds with membrane-destroying capabilities are cyclic peptide antibiotics (Fig. 32) such as subtilin from *Bacillus subtilis* and tyrocidin and gramicidin S, both derived from *Bacillus brevis*. Gramicidin S differs in structure and activity from the other gramicides (cf. later discussion). Surface-active antibiotics induce lysis of protoplast.



**Figure 30** Solubilization of a membrane protein (in the center) by tensides by generation of micelles.

## B. Formation of Artificial Pores

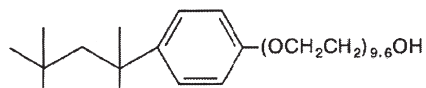
Transport antibiotics increase membrane permeability, i.e., selective permeability for certain substances. In some cases they are mobile, lipid soluble carriers (ionophores), which allow diffusion of certain ions through the membrane. In other cases these antibiotics form membrane pores, through which single ions permeate. These antibiotics play also a role in the energy transfer (cf. below) and are discussed in the following section.

## C. Valinomycin

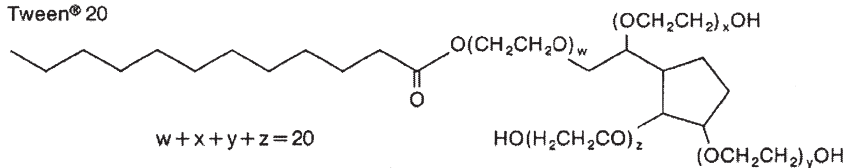
The antibiotic formed by *Streptomyces fulvissimus* is a cyclic peptide whose 12 ring components are arranged in alternating pairs of D and L configurations (Fig. 33). It is a threefold sequence of D-valine, D-hydroxy isovalerate, L-valine, and L-lactate, which are connected with each other by alternating ester and peptide bonds.

Valinomycin shows a high affinity for  $K^+$ , which is bound 1000-fold more strongly than  $Na^+$ . The antibiotic completely accommodates  $K^+$ , which is tightly held by hydrogen bindings. Complex formation requires alternating D and L pairs. A  $K^+$  ion fits exactly into the center of the valinomycin ring. This is not the case for other alkali metal ions. As a hydrophobic molecule, which does not contain any charged groups, the antibiotic can diffuse as ionophor through the double lipid layer of the membrane. In the presence of  $K^+$  the positively charged complex approaches the matrix as ionophor because of the negative membrane potential and releases its  $K^+$ . Mitochondria, for example, use the energy of the electron transport for  $K^+$  accumulation in the presence of valinomycin

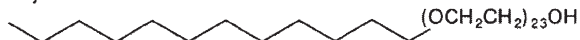
Triton® X-100



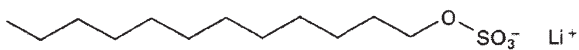
Tween® 20



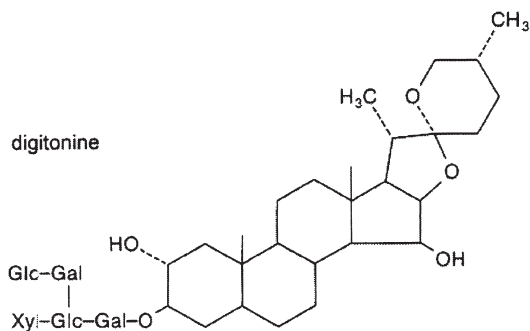
**Brij® 35**



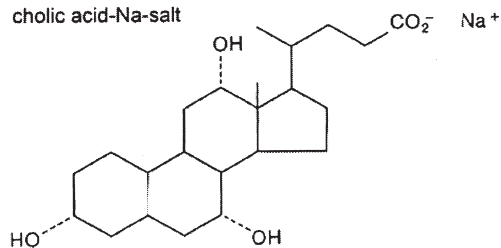
dodecylsulfate-Li-salt



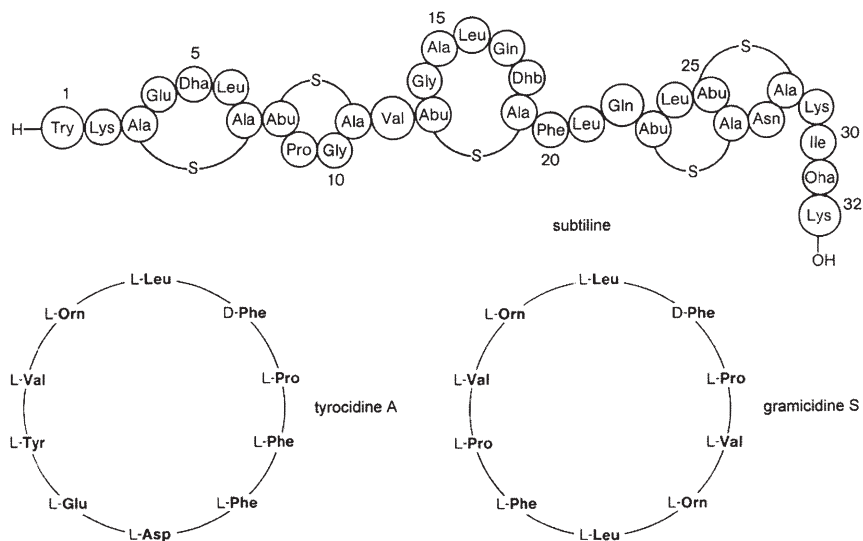
digitonine



cholic acid-Na-salt



**Figure 31** Tensides, which solubilize membranes.



**Figure 32** Cyclic peptide antibiotics.

at the expense of ATP. The antibiotic also binds to cytochrome c oxidase, thereby leading to an inhibition of the enzyme.

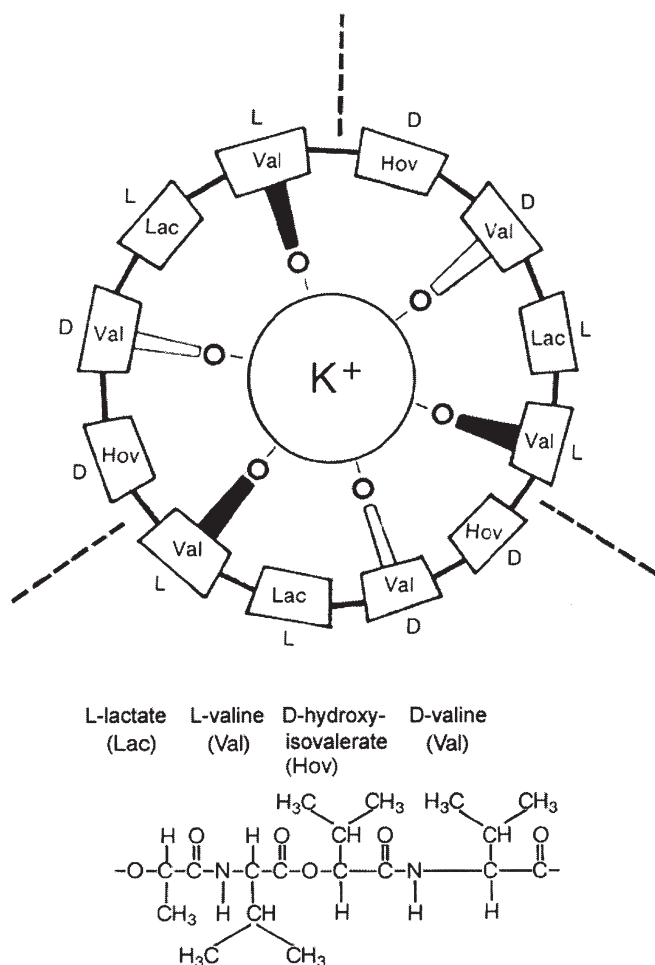
## D. Nigericin

The macrotetralide antibiotic from *Streptomyces violaceoniger* forms complexes with monovalent ions such as  $K^+$  (and to a minor extent with  $Na^+$ ) (Fig. 34). The transportation is carried out similarly to that of valinomycin.

## E. Gramicidin A

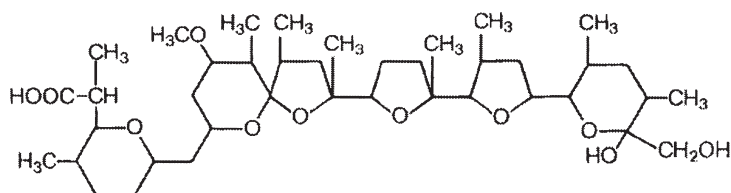
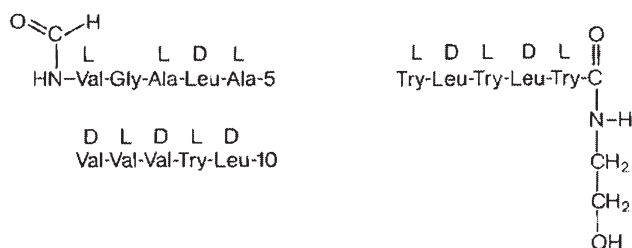
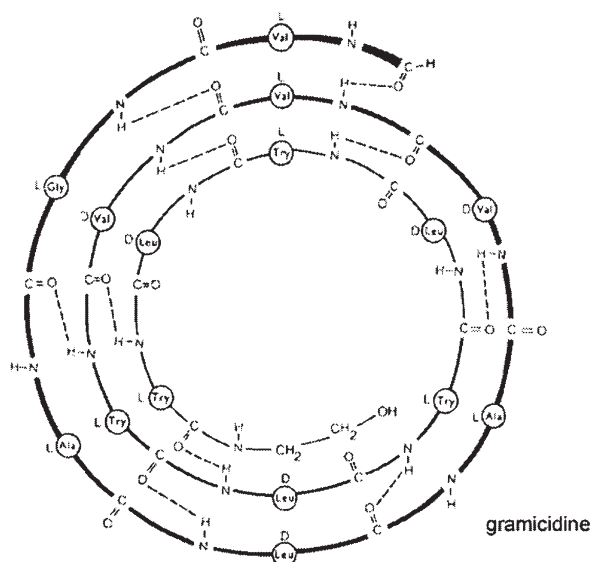
Gramicidin A is another transportation antibiotic from *Bacillus brevis*. This peptide, composed of 15 amino acid residues, is characterized by an alternating D and L configuration (Fig. 35).

Unlike the mobile carrier valinomycin, gramicidin forms a solid channel that crosses the membrane. This difference becomes manifested at low temperatures, which lead to solidification of the membrane: the speed of transport through the gramicidin pore is hardly impaired, whereas diffusion and thus transport of the valinomycin carriers are considerably inhibited.



**Figure 33** Valinomycin.

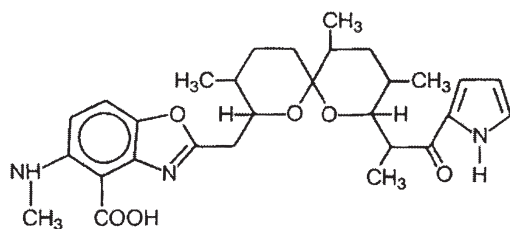
Pore formation by gramicidin A is based on the formation of a helical cylinder, which crosses the lipid membrane. It requires the association of two gramicidin monomers from opposite sides of the lipid double layer. The lipophilic groups point to the outside and the hydrophilic groups to the inside. The hydrophilic pore, which opens spontaneously and closes again within seconds, allows up to  $10^7$  monovalent cations per second to pass through. This process does not apply to bivalent cations or anions. The antibiotic does not discriminate between  $K^+$  and  $Na^+$ .

**Figure 34** Nigericin.**Figure 35** Gramicidin.

The transport speed through ionophores such as valinomycin is less than  $10^3$  ions per second.

A number of additional compounds can also bind divalent cations, particularly the carboxylic macrotetralide A 23187 (Fig. 36) from *Streptomyces chartreusensis*, which forms stable complexes with  $Mn^{2+}$ ,





**Figure 36** A 23187.

$\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  with relative affinities of 210:2,6:1. Two antibiotic molecules form a ringlike complex with the cation and function as a ionophore. Biological applications utilize the ionophore-induced change of the intracellular  $\text{Ca}^{2+}$  concentration. The antibiotic was used in the moss *Funaria* sp. to increase artificially the intracellular  $\text{Ca}^{2+}$  level. Even in the absence of cytokine stimulation of mitosis and bud formation results (72).

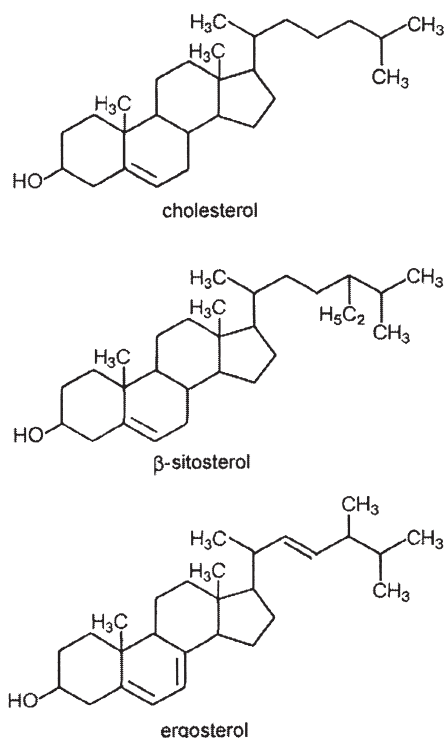
## F. Sterol-Dependent Permeability Changes

Sterols are important membrane components with a stabilizing function. Their removal leads to a permeability increase and in extreme cases to membrane disruption. Different organism groups differ in their sterol composition. Figure 37 shows important examples. In animal cells the C-27 compound cholesterol dominates. Higher plants contain different phytosterols (C-29 compounds) with sitosterol and stigmasterol as main components. In fungi, the C-28-sterol ergosterol is most abundant. However, arbuscular mycorrhizal fungi contain only minimal amounts of ergosterol. Here, the cholesterol campesterol (24-methylcholesterol) and 24-methylencholesterol (73) dominate. The proportion of sterols and their esters is particularly high in the plasmalemma.

Sterols play a pivotal role to ensure sufficient membrane fluidity, which is required for the activity of numerous membrane-bound enzymes. Interference with sterols and their synthesis therefore affects many membrane-bound enzymes.

## G. Polyene Antibiotics

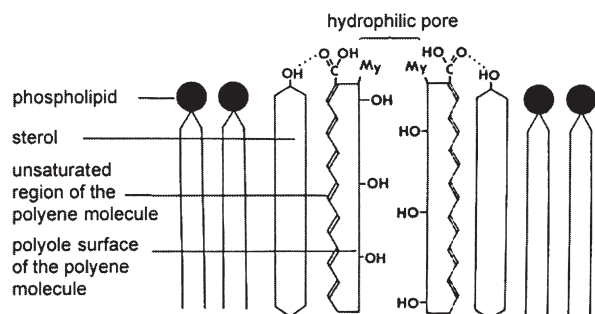
The macrolide polyene antibiotics selectively increase the permeability of sterol-containing membranes, frequently as a result of formation of a pore structure (Fig. 38).



**Figure 37** Sterols.

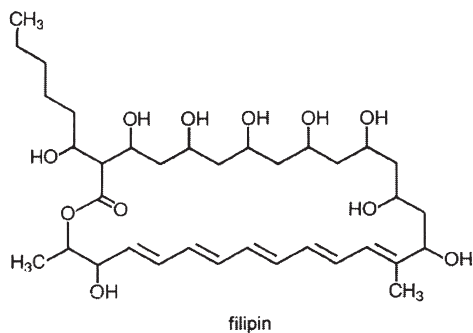
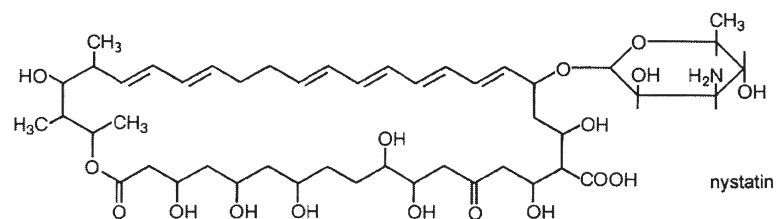
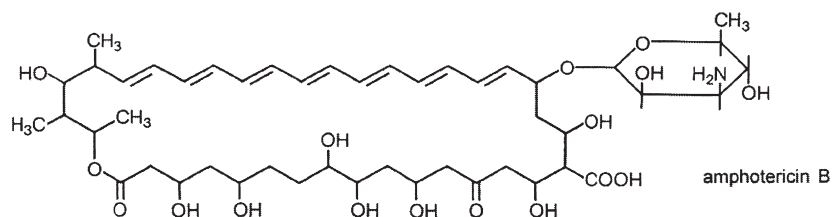
Since a number of polyenes show a specific affinity for ergosterol, their selective effect in fungi is intelligible. These antibiotics are intensively used for the therapy of human mycoses. Amphotericin B from *Streptomyces* species, as do all polyene antibiotics, consists of a large lactone ring substituted by several hydroxyl groups and a system of unsubstituted, all-trans conjugated double bonds (Fig. 39). A structural model is presented in Fig. 40. The biological action is based on hydrophobic interactions between an ergosterol molecule and the double-bond system of the antibiotic leading to the formation of a sterol polyene complex. The consequence is the release of sterols from the phospholipid interaction, resulting in the local change of the physical condition of the membrane, particularly the membrane fluidity. This presumably is the reason for increased proton permeability, as observed in the yeasts *Saccharomyces* and *Candida* spp. (76).

The antibiotic is used to treat systemic human mycoses, although toxic side effects (kidney damage, hemolysis) must be tolerated. Nyastin (Fig. 39),

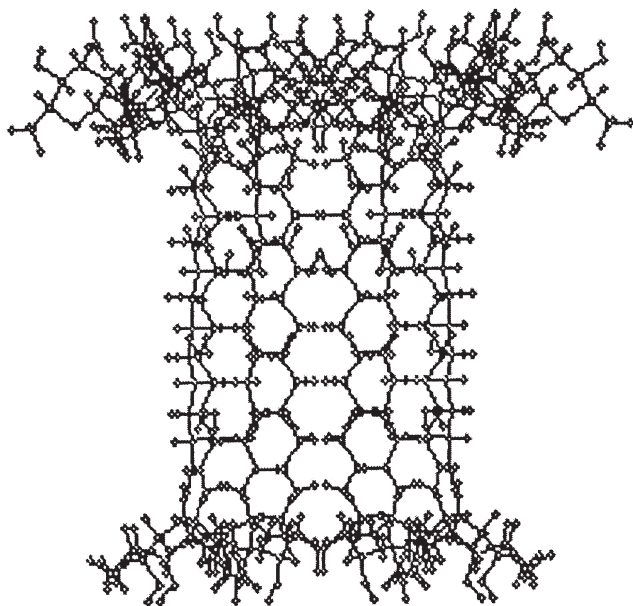


My: mycosamine

**Figure 38** Model of a polyene sterol pore. (From Ref. 74.)



**Figure 39** Polyene antibiotics.

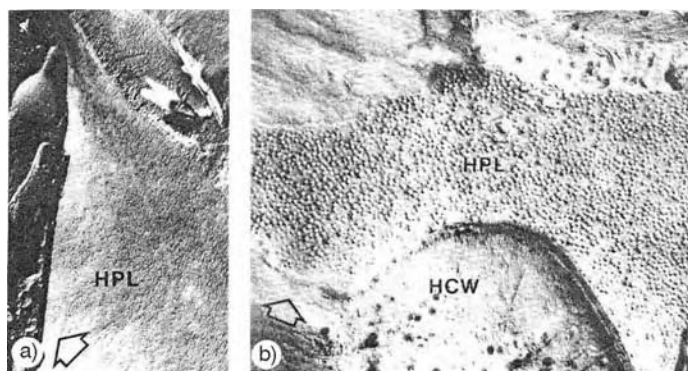


**Figure 40** Amphotericin B channel. (From Ref. 75.)

an important antimycotic compound from *Streptomyces noursei* and other *Streptomyces* species, is employed for the treatment of local mycoses (dermatoses). It induces the formation of nyastin-sterol pores followed by the disturbance of membrane functions. A nyastin-resistant mutant of *Aspergillus niger* shows an altered composition of the membrane lipids with considerably reduced amounts of sterol (77).

Filipin (Fig. 39) from *Streptomyces filipensis* binds specifically to cholesterol and more weakly to related sterols, e.g., fungal ergosterol. Filipin-sterol aggregates are generated in the hydrophobic domain of the membrane and can be visualized by electron microscopy as granular extrusions of the plasmalemma (Fig. 41). This technique can be used for detecting the sterol distribution in membranes (78). The main part of sterols can be assigned to the plasmalemma by the aid of filipin-sterol complexes. In addition, the outer membrane of chloroplasts contains sterols (79).

The action mechanism is different from that of polyene antibiotics described: it is unlikely that pores are formed. The high toxicity can be explained by membrane damage, which is due to disturbances of the lipid bilayer, which change the membrane permeability, as well as membrane fragmentation. Because of its effectiveness against many phytopathogenic



**Figure 41** Effect of filipin on mesophyll cells from beans: a, Control; b, filipin treatment (10  $\mu\text{g}/\text{ml}$ ) plasmalemma; 20- to 25-nm-large granula are distributed across the plasmalemma. HPL, HCW, cell wall. (From Ref. 78.)

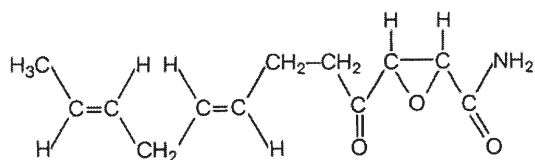
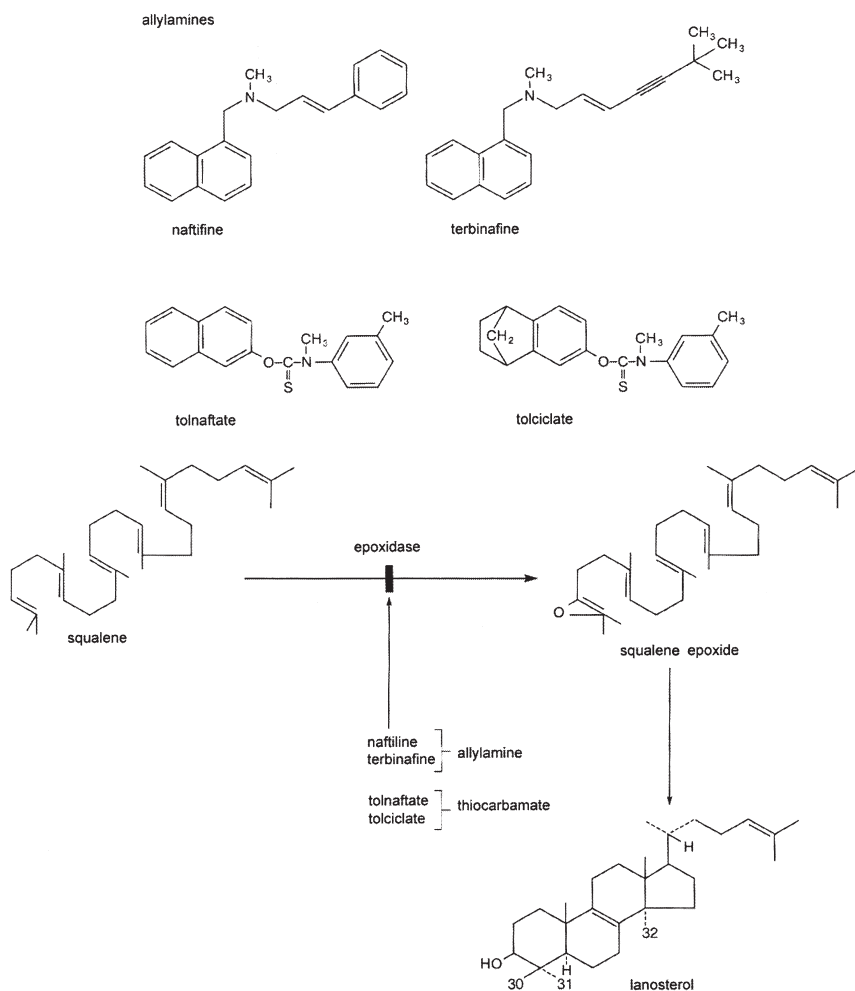
fungi, filipin has been repeatedly applied in plant protection, e.g., against the mildew of lima beans (*Phytophthora phaseoli*).

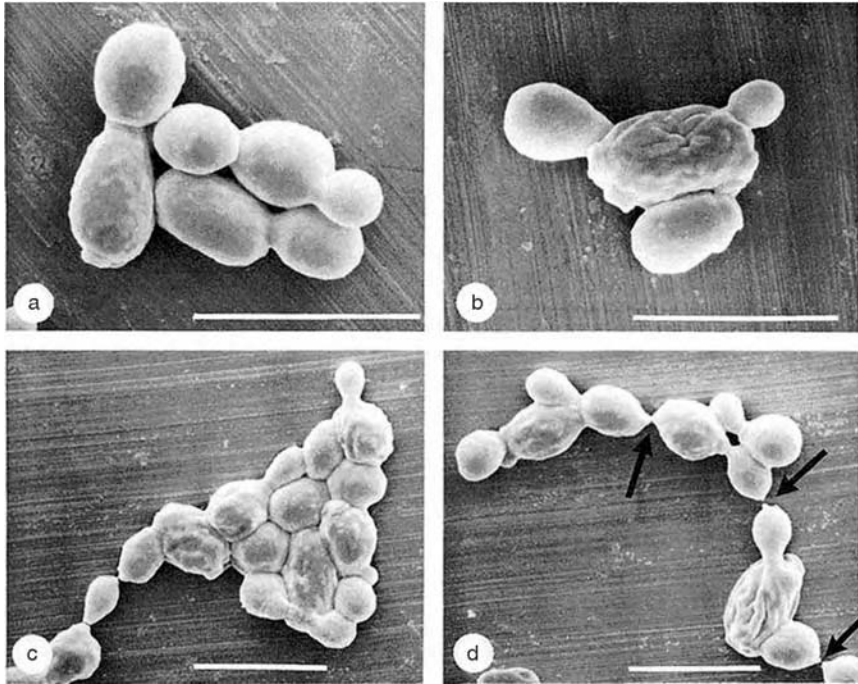
### XIII. INHIBITION OF MEMBRANE LIPID SYNTHESIS

Cerulenin, an antibiotic from the imperfect fungus *Cephalosporium caerulens*, is effective against fungi and some bacteria (Fig. 42). It blocks sterol synthesis by inhibition of the hydroxymethylglutaryl-coenzymeA (CoA) synthase as well as fatty acid synthesis by attack on the  $\beta$ -ketoacylthioester synthetase.

#### A. Inhibitors of the Ergosterol Biosynthesis

Damage of fungal membranes can be directly achieved by interference with ergosterol biosynthesis. Two main options are possible: (a) inhibitors of the squalene epoxidases as the allylamines naftifine and terbinafine and the thiocarbamates tolnaftate and tolciclate (Fig. 43) that interrupt the step from squalen to the squalen epoxide (cf. also Section 4). Fig. 44 shows the effect of naftifine on the yeast *Candida parapsilosis* including distortion (Fig. 44b), agglutination (Fig. 44c), and sticky cell connections (Fig. 44d, arrows). (b) inhibitors of the cytochrome P450-dependent  $14\alpha$ -demethylation of lanosterol block the step to ergosterol. A number of imidazole and triazole compounds are available. They are used both as pharmaceuticals and fungicides. Figure 45 shows

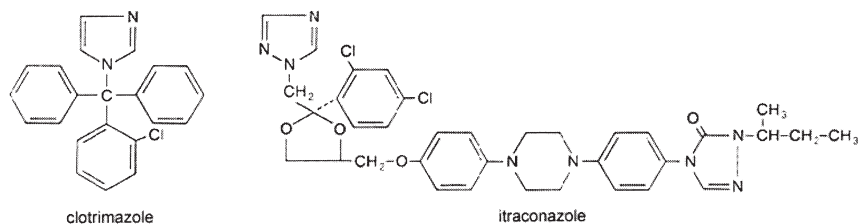
**Figure 42** Cerulenin.**Figure 43** Squalene epoxidase inhibitors and their mode of action.



**Figure 44** Effect of naftifine (50  $\mu\text{g/ml}$ ) on *Candida parapsilosis*: a, Control; b, cells treated after 2 h; c, d, after 24 h. Measuring lines, 5  $\mu\text{m}$ . (From Ref. 80.)

the imidazole clotrimazole and the triazole itraconazole as examples. Both compounds are used against pathogenic *Candida* species. Itraconazole binds to the cytosolic P450 isoenzymes in microsomes of *Saccharomyces cerevisiae* and *Candida albicans* and prevents demethylation. Defects of membranes and membrane-bound enzymes are the consequences, observed, for instance, in microsomes and mitochondria. In addition, direct influences on membrane fatty acids as well as inhibition of respiratory cytochromes are found.

The accumulation of  $14\alpha$ -methylsterols after blocking of the demethylation step leads to uncoordinated chitin synthesis, morphological changes, and cell lysis in fungi. Chitin is the main component of the primary septum, which separates bud and mother cell in yeasts such as *Candida albicans*. Its mycelial form requires chitin for the growth of the hyphal tips as well as for septum closure. This mechanism also explains damage in yeasts by azoles.



**Figure 45** Azoles as inhibitors of the lanosterol demethylation.

## B. Changes of Membrane Adenosine Triphosphatases

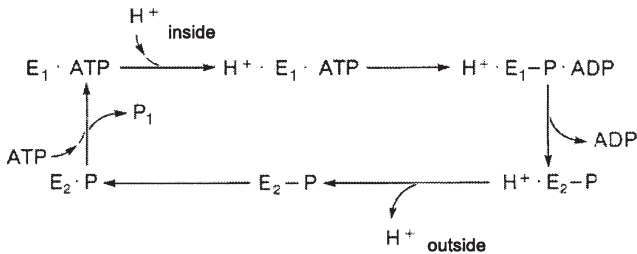
The membranes of the cell include a number of ion pumps. They are responsible for essential transport functions. The primary active transport process of plants and fungi is electrogenic proton transport, catalyzed by  $H^+$ -adenosine triphosphatases (ATPases) that function as proton pumps. The electrochemical  $H^+$  gradient is used for the cotransport of cations, anions, as well as sugars and amino acids. Furthermore  $H^+$ -ATPases are involved in the regulation of the cytoplasmic pH, opening of stomata, phloem loading, and cell elongation. By this means, an electrochemical potential, which is a prerequisite for metabolism and development in plants, can be maintained across membranes. In contrast to  $H^+$ -ATPases, which as ATP-powered pumps remove protons from the cytosol and thereby provide an electrochemical potential difference between apoplast and symplast (inside negative), the F-ATPases of chloroplasts and mitochondria (discussed later) inversely utilize the electrochemical proton gradient for ATP synthesis. Finally, the  $H^+$ -translocating inorganic pyrophosphatase (PPase) contributes to the proton transport to the vacuole.

## C. P-Adenosine Triphosphatases of the Plasma Membrane

The  $H^+$ -ATPase of the plasmalemma is a key enzyme that regulates membrane potential, nutrient uptake, and cytosolic pH value. This enzyme belongs to the P class of  $H^+$ -ATPases, which differs from other ATPases by its structure and function. Both  $Ca^{2+}$ -ATPase and  $Na^+/K^+$ -ATPase also belong to the P-ATPase group, which have the smallest size of all ATPases.

During ATP hydrolysis a covalent intermediate is formed by the enzyme involving the binding of the hydrolysed, terminal phosphate residue of ATP to an aspartate residue of the enzyme. Thus an aspartyl phosphate is produced. The enzyme exists in two conformations, the E1 and E2 stages. Therefore, these ATPases are also designated E1/E2-ATPases.





**Figure 46** Catalytic cycle of the P-ATPase. ADP, adenosine diphosphate; ATP, adenosine triphosphate; P-ATPase, plasma membrane H<sup>+</sup>-ATPase. (From Ref. 81.)

During the catalytic cycle E1 and E2 alternate. In the conformation E1 the transport side is directed to the cytoplasm and the enzyme has a high affinity for its substrate (e.g., H<sup>+</sup>) to be transported. In the conformation E2 the transport side is directed to the outside and the affinity for the substrate is low. The consecutive conformational changes are driven by the following events (Fig. 46):

1. Binding of ATP and the cation to be transported by the enzyme.
2. Hydrolysis of ATP to ADP and phosphate during phosphorylation of the aspartate residue.
3. Release of ADP to the medium.

The two last steps lead to the E2 state and the release of the cation to the outside. In the scheme the sequential course of the ATP-mediated transport of protons by fungal plasma membrane ATPases is presented as an example. The H<sup>+</sup> transport is characterized by a H<sup>+</sup>/ATP stoichiometry of 1: i.e., 1 H<sup>+</sup> is transported per hydrolyzed ATP. A detailed description is found in (81).

In the plasma membrane H<sup>+</sup>-ATPase is found in particularly high concentrations in the stomatal guard cells of higher plants, where the light-stimulated opening of stomata is controlled by H<sup>+</sup> extrusion. The creation of an electrochemical gradient drives K<sup>+</sup> uptake by K<sup>+</sup>-selective ion channels. This process together with simultaneous malate synthesis and/or Cl<sup>-</sup> uptake leads to a decrease of the intracellular water potential. Consequences are an influx of water, turgor increase, and finally opening of stomata.

In the shoot the enzyme is also abundant in all phloem cells, which are characterized by high transport activities connected with phloem loading. All other shoot tissues have considerably lower activities. Phloem loading results from a coupled H<sup>+</sup>-sucrose symport (1:1 stoichiometry) in sugar beet leaves. A sucrose-induced alkalization of the external medium

was noticed in isolated plasma membrane vesicles. In this case the P-ATPase-dependent  $H^+$  transport is carried out from the outside to the inside.

In the root P-ATPase is primarily found in epidermal cells including the root hairs and in the companion cells of the phloem. The enzyme has been examined particularly well in the plasma membrane of several yeasts. Amino acid import is coupled here to  $H^+$  extrusion.

As in the case of all enzymes that form a covalent phosphorylated enzyme intermediate, the phosphate analog orthovanadate ( $H_2VO_4^-$ ) also acts as a strong inhibitor. All processes listed previously, e.g., the light-stimulated increase of turgor in stomatal guard cells, are specifically inhibited by vanadate. On the other hand, the F-ATPases of mitochondria and chloroplasts as well as the V-ATPases of the tonoplast lack inhibition since respective intermediates are not formed.

Other inhibitors such as *N,N'*-dicyclohexyl carbodiimide (DCCD) (discussed later), diethylstilbestrol, octylguanidine, and sulfhydryl inhibitors block the plasmalemma enzyme nonspecifically. Mycotoxins such as zearalenon from *Fusarium* sp. or *Cercospora beticola* toxin are also effective. In contrast, the enzyme is not impaired by molybdate, nitrate, or oligomycin. Table 3 gives an overview of further inhibitors. Table 4 summarizes modulators of the  $H^+$ -ATPase of the plasmalemma. Syringomycin is a mixture of related lipodepsipeptides of the plant pathogen *Pseudomonas syringae*, pv. *syringae*, responsible for the brown spot disease of beans and other diseases. The stimulation of the  $H^+$ -ATPase of the plasmalemma is carried out by syringomycin activation of a membrane-bound protein kinase, which phosphorylates and activates the  $H^+$ -ATPase of the plasmalemma, but not of the other  $H^+$ -ATPases. This finally leads to the  $K^+$  efflux from the cell via a stimulation of the  $H^+/K^+$  antiport.

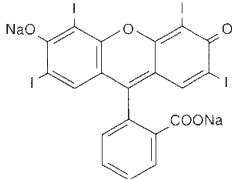
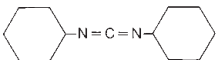
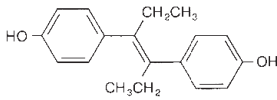
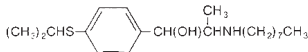
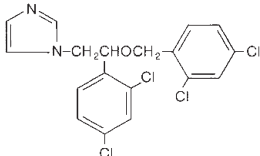
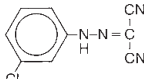
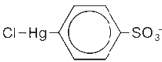
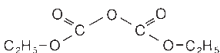
## 1. Fusicoccin

Fusicoccin (Fig. 47) is an activator of the  $H^+$ -ATPase of the plasmalemma. This diterpen glucoside from the imperfect fungus *Fusicoccum amygdali* acts as a wilting toxin and is responsible for pathological symptoms in almond and peach trees. The structurally similar cotylenine from *Cladosporium* sp. evokes physiologically comparable effects.

The intensive use of fusicoccins in plant physiological research started in 1971 with the discovery that fusicoccin dramatically stimulates cell growth. A detailed description is found in (82).

An application of fusicoccin immediately leads to  $H^+$  increase in the apoplast. This results from the activation of the plasma membrane

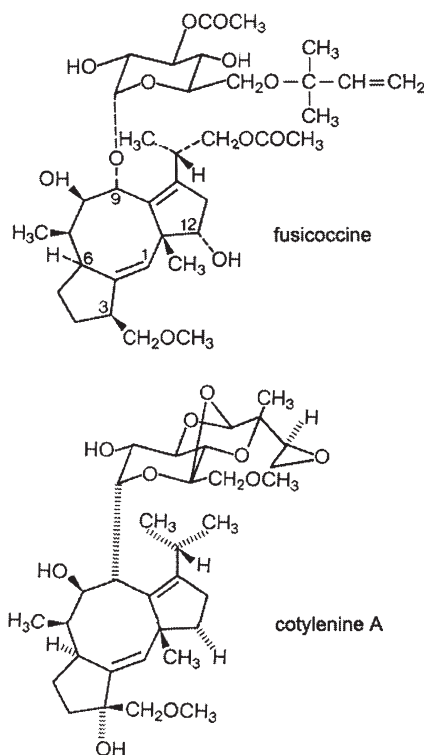
**Table 3** Inhibitors of Plasma Membrane  $H^+$ -Adenosine Triphosphatase

Compound	Structure	Note
Vanadate	$VO_4^{3-}$	Inhibition of reaction cycle after phosphate release
Erythrosin B		Inhibition of ATP binding, first step of reaction cycle
Heavy water <i>N,N'</i> -dicyclohexylcarbodiimide (DCCD)	$D_2O$ 	Kinetic effect Binding to a proteolipid subunit
Diethylstilbestrol		Unspecific inhibition
Suloctidil		Inhibition primarily of yeast P-ATPase
Miconazole		Inhibition primarily of P-ATPase of plants
Carbonylcyanide- <i>m</i> -chlorophenylhydrazone (CCCP)		Protonophore, abolition of pH gradient
<i>p</i> -Chloromercurylbenzol-sulfonic acid		Sulfhydryl reagent
Diethylpyrocarbonate (DEPC)		Histidine reagent, DEPC inhibition abolition by hydroxylamine
Gramicidin D		Linear gramicidin (polypeptide)
Octylguanidine		

**Table 4** Modulators of Plasma Membrane- $H^+$ -Adenosine Triphosphatase<sup>a</sup>

Compound	Effect
Fatty acid residues of phospholipids, e.g., 1-palmitoyl-2-oleoyl-phosphatidylserine, asolectine	Activation (depending on degree of saturation and chain length) through interaction of enzyme and phospholipids
$H^+$ (weak acid)	Activation: cytosolic pH modulation by proton pump
$K^+$ , $Mg^{2+}$	Activation at low concentrations
Light (blue light)	Stimulation at stomata
Auxin	Stimulation (probably indirect)
Frost	Increase of activity after light (reversible) frost damage, decrease of activity after heavy (irreversible) damage
Syngomycin (from <i>Pseudomonas syringae</i> , pv. <i>syringae</i> )	P-ATPase activity increased by activation of a membrane-associated protein kinase, which among other things phosphorylates and activates ATPase
Elicitors, e.g., arachidic acid	Receptor-bound elicitor inhibition of P-ATPase, which thereby induces phenyl-propane pathway and thus formation of phytoalexin
Fusicoccin	Activation (indirect via fusicoccin receptor)
Nectrosin-inducing peptides (from fungus <i>Rhynchosporium secalis</i> )	Activation (direct)
Calmodulin antagonists (chlorpromazine, trifluoperazine)	Activation
Lysolecithin	Activation
Platelet-activating factor (PAF)	Activation

<sup>a</sup>P-ATPase, plasma membrane  $H^+$ -adenosine triphosphatase.



**Figure 47** Fusicoccin and cotylenin, fungal toxins produced by *Fusicoccum* and *Cladosporium* spp., respectively.

$H^+$ -ATPase by changes in its C-terminal domain and the hyperpolarization of the plasma membrane. The following mechanism has been proposed: fusicoccin binds with high affinity to the 14-3-3 protein, a regulatory protein, which normally binds to a phosphorylated threonine residue of the C-terminal domain of the plasma membrane  $H^+$ -ATPase, but also other enzymes, such as nitrate reductase. However, fusicoccin-induced 14-3-3 binding occurs regardless of phosphorylation.

Table 5 summarizes a number of events that are stimulated by fusicoccin *in vivo*. Some effects of fusicoccin and auxin are similar, although the receptors involved are not identical. Distinct differences exist, for instance, in the reduction of phosphatidyl inositol, which is promoted by auxin, but not by fusicoccin (83).

The stimulation of the elongation growth by fusicoccin is in accordance with the acid growth theory: the secretion of protons to the cell wall area is triggered by activation of  $H^+$ -ATPase and the cell wall

**Table 5** In Vivo Effects of Fusicoccin

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Acidification of incubation medium (by $H^+$ transfer)
Hyperpolarization of membrane potential
Stimulation of substance transport
Absorption of $K^+$ and other cations
Absorption of $Cl^-$ and other anions
Active $Na^+$ release
Absorption of glucose, sucrose, and amino acids
Increase of metabolic activity
Respiration and dark $CO_2$ fixation (as malate)
Increase of pyruvate and glucose-6-phosphate level; decrease of $C_1/C_6$ ratio
Physiological effects
Stimulation of cell elongation growth
Stimulation of stomata aperture (antagonism to abscisic acid)
Acceleration of seed germination (antagonism to
abscisic acid and conditions that cause dormancy)

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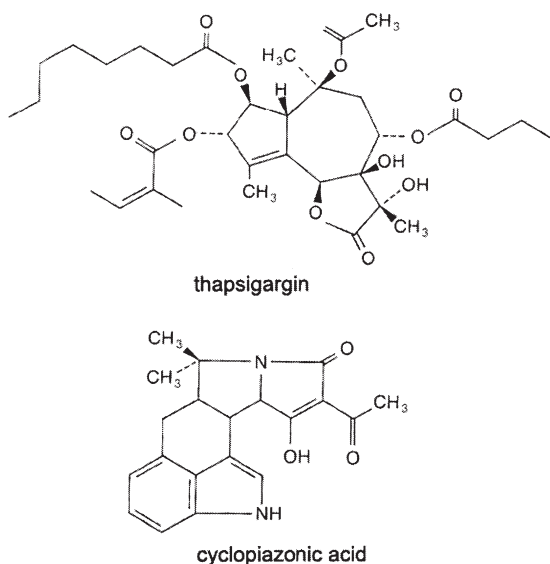
Source: Ref. 82.

becomes plastically deformed. However, fusicoccin and the cell-stretching hormone auxin increase the proton excretion by different action mechanisms. The receptors for the two compounds are different. Auxin does not influence the receptor binding of fusicoccin (84). As has been shown by cycloheximide experiments, the auxin-induced process depends on protein synthesis, whereas the fusicoccin-induced events do not (85).

The largest fusicoccin receptor quantities are found in the mung bean (*Vigna radiata*) hypocotyls, which show the strongest reactions to fusicoccin, but not in meristematic tissue (86). In addition to  $H^+$ -ATPase, fusicoccin activates the  $Ca^{2+}$ -ATPase of the plasma membrane (87), which as a primary ion pump removes  $Ca^{2+}$  from the cell (see later discussion).

#### **D. $Ca^{2+}$ -Adenosine Triphosphatase**

The  $Ca^{2+}$ -ATPases of the plasmalemma and the endoplasmic reticulum (ER) are in the class of P-ATPases. They are significantly involved in the active removal of  $Ca^{2+}$  from the cell and therefore in cytosolic  $Ca^{2+}$  homeostasis, which retains  $Ca^{2+}$  concentrations at low levels ( $<0.1 \mu M$ ). The crucial role of these enzymes is due to the fact that

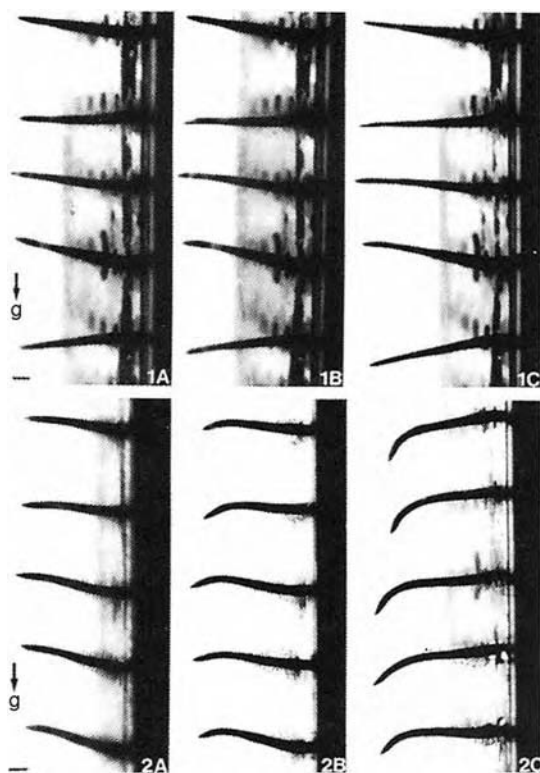


**Figure 48** Inhibitors of the  $\text{Ca}^{2+}$ -adenosine triphosphatase ( $\text{Ca}^{2+}$ -ATPase) of the endoplasmic reticulum (ER).

$\text{Ca}^{2+}$  serves as a second messenger in plants. A temporary modulation of the cytosolic  $\text{Ca}^{2+}$  concentration is involved in many signal communication processes, e.g., in phytochrome, and in gravity-controlled reactions, closure of stomata, as well as cold and salt stress.

A rise of the cytosolic  $\text{Ca}^{2+}$  concentration leads to the activation of the plasma membrane  $\text{Ca}^{2+}$  pumps, which quickly remove the  $\text{Ca}^{2+}$  from the cytosol. This effect is inhibited by vanadate and erythrosin B, an iodated fluorescein derivative (cf. Table 3). On the other hand, the sesquiterpene lactone thapsigargin (Fig. 48) from *Thapsia garganica* and the mycotoxin cyclopiazonic acid, an indole alkaloid from *Penicillium cyclopium* and *Aspergillus versicolor*, block the  $\text{Ca}^{2+}$ -ATPase of the ER. Aluminum fluoride ( $\text{AlF}_4^-$ ) inhibits the  $\text{Ca}^{2+}$ -ATPase of the plasma membrane and ER by interfering with the formation of the phosphoprotein intermediates.

Figure 49 shows the inhibition of the gravitropic reaction after treatment of cress roots with cyclopiazonic acid (77). This ER-bound enzyme is presumably involved in the signal transduction of the gravistimulus, which leads to a short-term rise of the  $\text{Ca}^{2+}$  concentration. Calmodulin stimulates this enzyme. All calmodulin antagonists (e.g., the imidazole compound calmidazolium) therefore indirectly inhibit this enzyme.



**Figure 49** Influence of cyclopiazonic acid ( $20\text{ }\mu\text{M}$ ; pre treatment, 2 h, upper row) on the gravitropic reaction of cress roots. Lower row, Control. A, Time 0 min; B, after 40 min; C, after 120 min; g, direction of the gravity vector; Measuring bar, 1 mm. (From Ref. 88.)

### E. V-Adenosine Triphosphatases of the Tonoplast

The vacuole is a multifunctional compartment. The acidic cell sap contains a variety of substances, which play a role in the regulation of osmosis, storage of a vast variety of solutes, and defense against pathogenic organisms. Import from the cytosol requires in most cases an electrochemical potential across the tonoplast membrane. The electrochemical potential in yeasts is  $180\text{ mV}$  (inside positive) and is generated by a V-ATPase located in the tonoplast membrane.

In addition, a pyrophosphatase is involved (discussed later). The function of an amino acid/ $\text{H}^+$ - and a  $\text{Ca}^{2+}$ -antiporter as well as the function of a  $\text{K}^+$ -channel depends on the acidification of the vacuole.



Although V- and F-ATPases are derived from a common gene and bear structural and functional similarities, V-ATPases are characterized by several unusual properties (cf. also Table 6): the enzyme complex (Fig. 50) is bigger than that of the other ATPases. No phosphorylated intermediate product is formed during the catalytic reaction cycle. Therefore, no vanadate inhibition occurs. Unlike the F-ATPases of mitochondria and chloroplasts, the enzyme is not inhibited by oligomycin or azide. A specific inhibitor, however, is  $\text{NO}_3^-$ . But the inhibition constant is very high ( $K_i = 5\text{--}10\text{ mMol/l}$ ). Tributyltin, DCCD, and diethylstilbestrol (DES) are used as nonspecific inhibitors. The iodinated fluorescein derivatives erythrosin B and rose bengal also block the enzyme. Cold treatment decreases the activity by release of peripheral subunits from the catalytic site and partial disassembly of the ATPase complex.

V-adenosine triphosphatase is most effectively stimulated by  $\text{Cl}^-$ . This mechanism is used by the cell for direct regulation by the cytosolic  $\text{Cl}^-$  concentration.  $\text{Mg}^{2+}$  is required for the activity.  $\text{C}_4^-$  (e.g., malat) and  $\text{C}_5^-$ -dicarboxylic acids are also stimulating.

## F. Pyrophosphatases


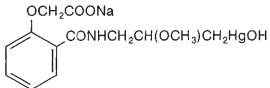
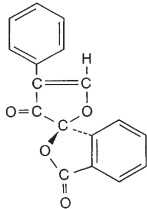
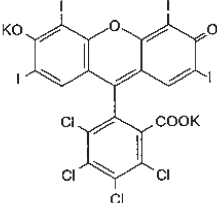
In addition to V-ATPase, an inorganic pyrophosphatase ( $\text{H}^+$ -PPase) is involved in the proton transport into the vacuole. It requires  $\text{K}^+$  and  $\text{Mg}^{2+}$  on the cytosolic side.  $\text{H}^+$ -pyrophosphatase is a tonoplast-bound enzyme, which splits pyrophosphate into two molecules of phosphate. The free energy is used for proton translocation and thus indirectly for the import of metabolites into the vacuole.  $\text{Mg}_2\text{PP}_i$  serves as substrate. This complex also functions as a noncompetitive inhibitor at the same time. The pyrophosphatase can be inhibited by fluoride and *N*-ethyl maleimide.

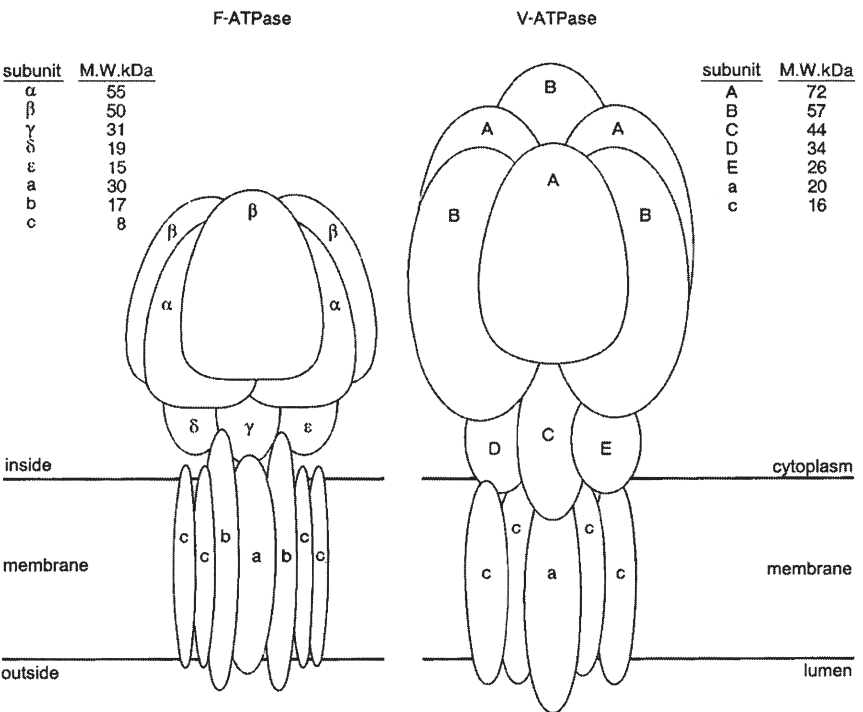
## XIV. ATTACK ON ION CHANNELS

Ion channels are integral components of all membranes, which permit the passage of ions through a membrane. Unlike carrier systems (discussed later), they are characterized by high turnover rates (approximately  $10^6\text{--}10^8\text{ s}^{-1}$ ). Patch-clamp technology (90) has contributed essentially to a better understanding of ion channels, since the activity of single ion channels can be analyzed by this technique.

Ion channels can be regarded as dynamic ion transport systems coupled via membrane electrical activities. Not only do they influence membrane potential by the ionic currents they mediate, but their activities

**Table 6** Inhibitors of Tonoplast H<sup>+</sup>-Adenosine Triphosphatase

Compound	Structure	Note
Nitrate	$\text{NO}_3^-$	Effect that leads to dissociation of peripheral polypeptides of integral membrane complexes of enzyme
<i>N,N'</i> -dicyclohexylcarbodiimide (DCCD)	cf. Tab. 4	Binding to a proteolipid subunit
<i>N</i> -ethylmaleimide (NEM)		Sulfhydryl reagent (permanent)
Diethylstilbestrol (DES)	cf. Tab. 4	Synthetic estrogen
<i>p</i> -chloromercurybenzoic acid (PCMB)		Sulfhydryl reagent (nonpermanent)
Mersalyl		
Bafilomycin		Macrolide antibiotic of <i>Streptomyces</i> sp.
7-Chloro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl)		
2,3'-Dialdehyde derivative of adenosine triphosphate (ATP)		
Fluorescamine		Reaction with primary amines, inhibition of proton translocation, no inhibition of ATP hydrolysis
Tributyltin	$(\text{C}_4\text{H}_9)_3\text{Sn}^+$	
Erythrosin B	cf. Tab. 4	Fluorescein derivative
Rose bengal		Fluorescein derivative



**Figure 50** Basic structure of the F- and V-ATPases. (From Ref. 89.)

can also be regulated by the membrane potential. Historically ion channels are divided into four groups according to the gating mechanism: ligand-gated, voltage-gated, stretch-activated, and light-activated.

Ligand-gated ion channels bind intracellular second messengers that provide the essential link between external stimuli and specific intracellular responses (91). Moreover, additional modulations by ATP or protons allow the channels to sense changes in energy status or acid metabolism, respectively (92). Voltage-dependent channels appear optimally suited for electrical signal transmission via membrane depolarization (e.g., through action potentials) and/or for signal transduction in response to changes in membrane potential. For instance, models that consider the coupling between membrane potential and voltage-dependent  $\text{Ca}^{2+}$  channels suggest that these are engaged in intracellular signaling.

Ion channels are also involved in membrane voltage stabilization, which is critical for maintaining ionic gradients and nutritional ion fluxes. Stretch-activated ion channels serve as additional specific transmembrane “receptors” coexisting with other cellular volume-sensing mechanisms.

Light-activated channels are in fact ligand-gated, although a precise indication of the ligands is not yet possible because the process of light signal transduction remains unclear. These channels are distinguished particularly by the special importance of light stimuli in plant signaling processes (93).

The electrical potential across a cell membrane is crucially influenced by the opening or closing of the ion channels. If the opening of an ion channel facilitates the passage of an ion, the net flux of this ion either into the cell or out of the cell is determined by the balance of two forces: one force results from the concentration gradient of this ion across the membrane;  $K^+$ , for instance, which is present in higher concentrations in the cell flows out. The second force is an electrical one. If the interior of the cell is negatively charged compared to the outside, positively charged ions such as  $H^+$  and  $K^+$  enter. As soon as the channel opens, these forces trigger a net influx of the respective cation. The electrical potential of the cell becomes more positive; depolarization takes place.

It is becoming increasingly apparent that the activity of a channel may depend on the developmental and metabolic stage of the cell. Moreover, regulation of ion channels relies not only on the channel proteins themselves, but also to a great extent on regulatory polypeptides, such as auxiliary  $\beta$ -subunits, cytoskeletal components, 14-3-3 proteins, phosphates, kinases, and G proteins (94).

Jan and Jan (95) (1997) reviewed receptor-regulated ion channels in excitable and nonexcitable animal tissue (G-protein-gated and cyclic guanosine monophosphate- [cGMP]-gated  $K^+$  channels; voltage-gated  $K^+$ ,  $Na^+$ ,  $Cl^-$ ,  $Ca^{2+}$  channels; voltage-insensitive  $Ca^{2+}$  channels;  $Ca^{2+}$ -activated  $K^+$  channels; ligand-gated  $Ca^{2+}$  channels). The activities of these channels are sensitive to external and internal signals that are mediated by receptors for hormones and transmitters. There are also plant-derived elicitor-specific receptors, which are closely coupled with plasma membrane ion channels important for signal transduction in plant cells (96, 97). Studies on receptor-regulated ion channels suggest that they too are gated via G proteins, either by direct protein-protein interaction or indirectly by kinase cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), protein kinase c (PKC)-phosphatase cascades or second-messenger binding ( $Ca^{2+}$ , inositol-1,4,5-triphosphate [ $IP_3$ ], cyclic guanosine monophosphate (cGMP), cAMP). A growing body of evidence indicates that G proteins, second messengers, and phosphorylation-dephosphorylation processes mediate various plant responses through ion channel and other transport system regulation. A comprehensive survey of ion channel classification hinged on both the type of transported ion and the corresponding gating mechanism was published in 2000 by Krol and Trebacz (93).

## A. Cation Channels

### 1. $K^+$ Channels

Potassium is among the main nutrients of the plant. The cytosolic concentrations are about 100 mmol/l.  $K^+$  as an osmotically active regulator accumulates in the vacuole, reaching concentrations up to 200 mmol/l, and in open stomatal guard cells up to 500 mmol/l. A negative membrane potential in the cytosol in the range of  $-120$  to  $-250$  mV drives  $K^+$  uptake. This membrane potential is generated by an electrogenic P-ATPase in the plasmalemma (discussed previously). Specific ion channels as well as  $K^+/H^+$  symporters are available for the passive  $K^+$  influx driven by the electrochemical gradient. These channels have to be distinguished from  $K^+$  efflux channels, which, for instance, allow the decrease of the osmotic potential for the closure of stomata.

The best characterized  $K^+$  channels of plants and fungi are voltage-sensitive channels. In this case a voltage change exerts an electrostatic force on the voltage sensors and leads to conformational changes and finally to channel opening; Fig. 51 shows a model.  $K^+$  channels play an important role with respect to growth, osmoregulation, cell movement, and mineral nutrition.

$K^+$  influx channels ( $I_{K, in}^+$ ) of the plasmalemma were initially detected at stomatal guard cells (99). An opening takes place at membrane hyperpolarization (at values below  $-100$  mVs). The  $K^+$  influx increases the osmotic potential.

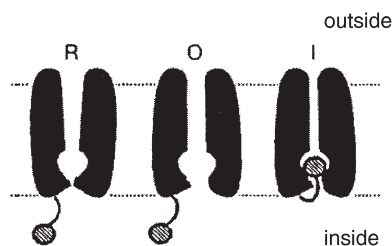
Approximately 200  $K^+$  influx channels per cell are found in mesophyll cells of oat leaves. These channels are opened at membrane voltages below  $-50$  mVs. This process is inhibited by low concentrations of  $Cs^+$  and  $Na^+$  (100), as well as by increase of cytosolic  $Ca^{2+}$  concentration.

Molecular studies showed that the channel consists of four subunits. Each subunit consists of six transmembrane segments (S1–S6). The voltage sensor lies within S4 and the pore between S5 and S6. A cyclic nucleotide binding site and an anchorage site for the cytoskeleton are located at the carboxyterminal end (101).

$Al^{3+}$  ions selectively block the influx, but not the efflux channels. This explains why  $Al^{3+}$  inhibits stomatal opening, but not closing.

A specific inhibition is achieved by tetraethylammonium (TEA), which binds to a threonine and a glutamine residue at the outer rim of the pore channel, as well as by 4-aminopyridine, quinine, charybdotoxin, and  $Ba^{2+}$  ions.

$K^+$  influx channels are also regulated by GTP binding proteins (G proteins). An activation by GTP leads to a decline of the  $K^+$  influx. Hints for a G protein regulation arise from examinations with nonhydrolyzable



**Figure 51** Ball and chain model of a voltage-sensitive  $K^+$  channel. Left, Resting stage; middle, open; right, inactivated. During opening of the channel the receptor becomes accessible as a so-called inactivation channel. (From Ref. 98.)

GTP and GDP analogs. Guanosine-5'-o-(2-thiodiphosphate) ( $GDP\beta S$ ) which blocks G proteins in a GDP-bound inactive form, increases the  $K^+$  influx. Guanosine 5'- $\gamma$ -triphosphate ( $GTP\gamma S$ ), which blocks G proteins in a GTP-bound inactive form, causes the opposite effect. The corresponding adenosine analogs show no effect at all (102).

$K^+$  efflux channels ( $I_{k, out}^+$ ) of the plasmalemma play a particular role in the stomata closure and the turgor decrease of motor cells in leave pulvini. The depolarization of the membrane potential, e.g., in stomatal guard cells, by increased abscisic acid and  $Ca^{2+}$  concentration leads to the opening of the channel and the  $K^+$  efflux. This process triggers a rehyperpolarization of the membrane potential together with an opening of  $Cl^-$  influx channels (103) (discussed later).

G proteins are also involved in the regulation of  $K^+$  efflux channels (104). The activation takes place at a depolarization of  $-47$  mVs (inside) of the plasma membrane to potentials between  $-15$  and  $+85$  mVs in mesophyll protoplasts of *Vicia faba*. This process can be inhibited by GTP analogs, by increase of cytosolic  $Ca^{2+}$  concentrations, as well as by cholera toxin. This toxin ADP-ribosylates the guanine nucleotide-binding regulatory  $G_s$  protein.

Different  $K^+$  channels are also found in tonoplasts. They can be blocked by TEA.

## 2. $Ca^{2+}$ Channels

The intracellular  $Ca^{2+}$  homeostasis is maintained by the coordinated activity of  $Ca^{2+}$ -ATPases,  $Ca^{2+}$  channels, and  $Ca^{2+}/H^+$  antiporters.  $Ca^{2+}$  influx channels of stomatal guard cells have been examined in most detail. The influx channels are activated by abscisic acid. They trigger a  $Ca^{2+}$  influx, the first step in the signal chain, which leads to stomatal closure (105).

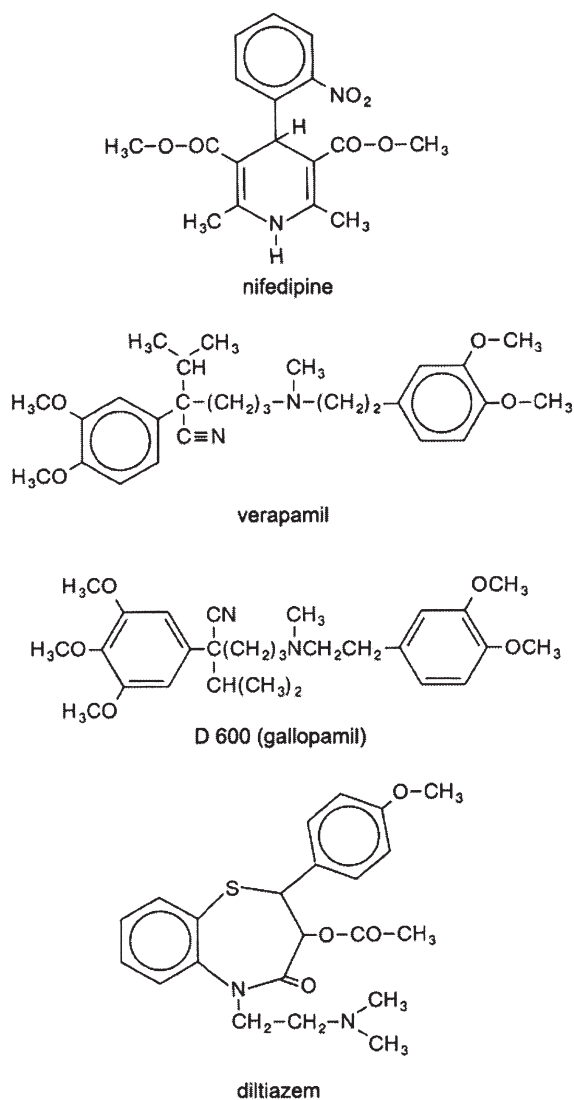
As is the case with the  $K^+$  channels, there are different classes of  $Ca^{2+}$  channels. They can be differentiated by their physical and pharmacological properties. At least three classes of voltage-sensitive  $Ca^{2+}$  channels exist: channels that are inhibited (a) by dihydropyridines such as nifedipine, (b) by phenylalkylamines such as verapamil or D 600, and (c) by benzothiazepines such as diltiazem (Fig. 52). The binding sites are presumably receptors for several phytotoxins, too. Further important  $Ca^{2+}$  channel blockers are  $La^{3+}$ ,  $Gd^{3+}$ ,  $Co^{2+}$ , and  $Ni^{2+}$ . The effect of these compounds on plants is not exclusively limited to  $Ca^{2+}$  channels (106).

Strong morphogenetical effects of  $Ca^{2+}$  channel blockers are observed where an intensive  $Ca^{2+}$  influx into the cell takes place, e.g., in pollen tubes and moss protonemata, which swell up or branch if the tip is treated. A  $Ca^{2+}$  influx is observed during differentiation of vessels as well as in phototaxis of algae. Both processes are impeded in the presence of inhibitors.

The vacuole represents the largest store for  $Ca^{2+}$  in the cell. There concentrations range between 1 and 10 mmol/l. Therefore, it is not surprising that different  $Ca^{2+}$  channels are found in tonoplasts whose opening induces a  $Ca^{2+}$  efflux from the vacuole. They include an inositol-1,4,5-triphosphate ( $IP_3$ )-sensitive channel, which is blocked by the glycosaminoglycan heparine as well as by 8-[*N,N*-dieththylamino]octyl-3,4,5-trimethoxybenzoate-HCl (TMB 8). The  $IP_3$  functions as a second messenger. It is released from the phospholipid membrane of the plasmalemma by light and hormones.

A voltage-sensitive  $Ca^{2+}$  channel, which is only inhibited by the lanthanide  $Gd^{3+}$  and to a lower extent by  $Zn^{2+}$ , shows other characteristics. This  $Ca^{2+}$  channel is not sensitive to  $IP_3$ . The channel is opened at a positive trans-tonoplast membrane potential (107).  $Ca^{2+}$  transport systems in plants are summarized in Fig. 53 (108), which explains mechanisms crucial for understanding the effects of different biological stimuli as well as pollutants. Complex phenomena such as  $Al^{3+}$  and NaCl toxicity have to be considered in the context of disturbed  $Ca^{2+}$  homeostasis.

Fast (primary) effects of  $Al^{3+}$  manifest themselves within seconds to minutes. Long-term (secondary) effects, however, can be detected only after several hours. The latter mainly cause an inhibition of root growth (mitosis and cell elongation). Primary effects of  $Al^{3+}$  occur on the apoplastic side of the plasma membrane (109), where  $Ca^{2+}$  uptake is blocked by the inhibition of  $Ca^{2+}$  channels. This mechanism may also prevent the increase of the cytosolic  $Ca^{2+}$  level, a prerequisite for the spindle formation during the prophase as well as for the metaphase-anaphase

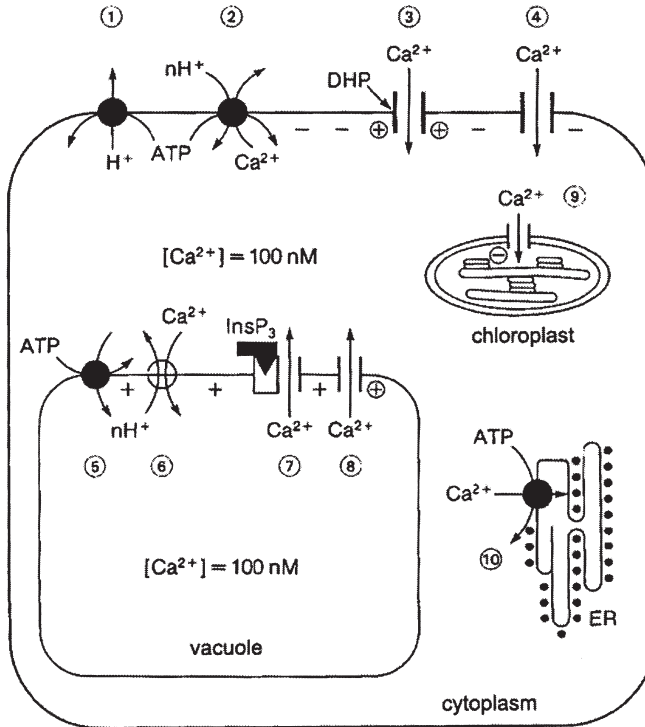


**Figure 52**  $\text{Ca}^{2+}$  channel blockers.

transition. Therefore, damage occurs when  $\text{Al}^{3+}$  is present only in the apoplast space. A detailed discussion of Al toxicity is found in Lüttge and Clarkson (110).

NaCl toxicity is based on an interference with  $\text{Ca}^{2+}$  homeostasis (111). Except for osmotic damages, which cause a water deficit by  $\text{H}_2\text{O}$





**Figure 53** Survey of plant transport systems that are involved in cytosolic  $\text{Ca}^{2+}$  homeostasis: 1, Plasmalemma  $\text{H}^+$ -ATPase; 2,  $\text{Ca}^{2+}$ -ATPase; 3 and 4,  $\text{Ca}^{2+}$  channels of the plasmalemma; 5,  $\text{H}^+$ -ATPase of the vacuole; 6,  $\text{Ca}^{2+}/\text{H}^+$  antiporter; 7 and 8,  $\text{Ca}^{2+}$  channels of the vacuole; 9,  $\text{Ca}^{2+}$  channels of chloroplasts; 10, ER  $\text{Ca}^{2+}$ -ATPase. DHP, dihydropyridine; ATP, adenosine triphosphate; ER, endoplasmic reticulum; ATPase, adenosine triphosphatase. (From Ref. 108.)

efflux according to the water potential gradient,  $\text{Na}^+$  reduces the binding of  $\text{Ca}^{2+}$  to the plasmalemma, inhibits the  $\text{Ca}^{2+}$  influx at an increased efflux, and depletes the intracellular  $\text{Ca}^{2+}$  sources (for instance, the vacuole and the ER). The  $\text{Na}^+$  uptake of the cell is already compensated for to a certain degree in the root by an active efflux from the cell as well as the accumulation in the vacuole. If these transport capacities are exhausted,  $\text{Na}^+$  ions finally reach the shoot axes and the meristems of young leaves via the xylem. These organs are the most sensitive ones in plants and immediately stop growth. Growth of the leaf area is primarily affected.

## B. Nonselective Cation Channels

Increasing evidence has emerged over the past decade of the existence of plant cation channels that are not very selective for cations. Termed *nonselective cation channels* (NSCCs), they have been extensively reviewed by Demidchik and associates (112).

In plants NSCCs are a large, heterogeneous group of channels. Generally, they show a high preference for cations over anions, but a low selectivity among monovalent cations under a wide range of ionic conditions, although they are not strictly nonselective. These channels usually have a permeability similar to that of a wide range of monovalent cations. Many of them do not, or poorly pass  $K^+$ - and  $Na^+$ -selective channels. They show  $K^+ : Na^+$  selectivity ratios between 0.3 and 3, although not exclusively. Nevertheless, NSCCs that have more pronounced discrimination between  $K^+$  and  $Na^+$  discriminate only poorly between other alkali metal cations. Some NSCCs are permeable to large cations such as  $Tris^+$ ,  $TEA^+$ , and  $choline^+$ . Nevertheless, a significant group of NSCCs are insensitive to organic inhibitors of  $Ca^{2+}$ -selective and  $K^+$ -selective channels such as verapamil, nifedipine, and  $TEA^+$ .

However, caution in using inhibitors is advised. For example, channel-like nonselective ion transport can be induced through plant plasma membranes by application of verapamil (113) and quinine. No specific inhibitors are known for NSCCs, and finding any seems unlikely, considering the diversity of this class of channels.

There is a range of NSCCs in several plant membranes controlled by parameters such as voltage,  $Ca^{2+}$ , and glutamate. They permit movement of monovalent or divalent cations and appear to constitute the primary pathway of  $Ca^{2+}$  uptake and signaling in plants. The NSCCs are involved in a wide range of processes, including low-affinity nutrient acquisition and allocation, turgor control, intracellular transport, and signaling. The low selectivity of these channels also suggests their involvement in uptake of toxic cations, notably  $Na^+$ .

Two examples of NSCCs refer to plant endomembranes (112), ER membranes and symbiosome membranes.

Similarly to vacuoles the ER can serve in  $Ca^{2+}$  storage, releasing  $Ca^{2+}$  as part of intracellular signaling pathways. Several studies using the planar lipid bilayer technique revealed the existence of  $Ca^{2+}$ -permeable ion channels in the ER membranes of cells that respond to mechanic stimuli. Klüsener and colleagues (114, 115) characterized a channel from ER of the touch-sensitive tendrils of *Bryonia dioica*. A second channel was characterized from endomembranes (most likely ER) of root tips from garden cress (*Lepidium sativum*). In each case, the permeability ratio

$\text{Ca}^{2+}:\text{K}^{+}$  of the channels was low (6.6 and 9.4, respectively), suggesting that these channels are NSCCs. Gating was voltage-dependent (inward-rectifying) and controlled by the  $\text{Ca}^{2+}$  gradient across the ER membrane. The *Bryonia* sp. channel was inhibited by verapamil and lanthanides as well as by  $\text{H}_2\text{O}_2$ . The *Lepidium* sp. channel was sensitive to lanthanides, but not to verapamil, and was strongly blocked by the  $\text{Ca}^{2+}$ -ATPase inhibitor erythrosin B. This suggests that the channel could consist of the uncoupled transmembrane domain of a  $\text{Ca}^{2+}$ -ATPase (116).

In nodulated legumes, the symbiotic  $\text{N}_2$ -fixing bacteria occur in the plant cell cytosol in a symbiosome enclosed within a plant-derived membrane. Exchange of fixed nitrogen ( $\text{NH}_4^+$ ) via an NSCC and reduced carbon between the peribacteroid space and the plant cytosol takes place across this membrane (117). The channel appears to be intrinsically voltage-insensitive but gated by divalent cations. Millimolar concentrations of  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  on each side of the membrane eliminated cation efflux from that side and caused influx to become time-dependent. This finding suggests that  $\text{NH}_4^+$  currents are inwardly rectified in vivo by cytosolic  $\text{Mg}^{2+}$  blockade. Verapamil added to the bacteroid side inhibits cation influx to the plant cytosol with a  $K_d$  of  $2.6\text{ }\mu\text{M}$ , but there is no evidence of divalent cation permeation of the channel (118).

The NSCCs have several advantages over highly selective ion channels. One advantage is that they can transport different ions in both directions. For instance, the depolarization caused by  $\text{Ca}^{2+}$  entry through NSCCs could be offset by  $\text{K}^{+}$  efflux via the same channel. The NSCCs interact less strongly with permeant cations and thus may be able to maintain uptake of the target cation in the presence of cations that block more selective channels. This capacity would be an advantage in fluctuating soil solutions, where  $\text{K}^{+}$  uptake must be maintained in the presence of  $\text{Cs}^{+}$ , for instance, or  $\text{Ca}^{2+}$  in the presence of other divalent cations. These channels also provide a passive route for uptake of cations for which high selectivity may not be possible (for instance,  $\text{NH}_4^+$  or  $\text{Mg}^{2+}$ ). Relatively indiscriminant cation transport may be an advantage for turgor-controlled ion channels, which must mediate rapid exchange of solutes (112).

### C. Mechanosensitive Ion Channels

In numerous cases cells need information about changes in volume and pressure, e.g., in stomatal guard cells, where turgor changes determine the degree of stomatal opening, or in cells with tip growth such as root hairs, pollen tubes, or fungal hyphae. Mechanosensitive ion channels, which are activated or inactivated by membrane tension, are an elegant solution.

Three different channels activated by tension were detected in stomatal cells by the aid of patch-clamp technology. These channels are permeable for  $K^+$ ,  $Ca^{2+}$ , and  $Cl^-$  ions and completely differ in their properties from the corresponding nonmechanosensitive channels (119). There are also sensors for turgor, which can serve as safety valves for excess pressure. A channel activated by membrane tension for  $Ca^{2+}$  and  $K^+$  as well as a smaller channel for  $Mg^{2+}$  were found in hyphal tips of the oomycete *Saprolegnia* sp. (120). An ion gradient can probably be generated from the hyphal tip in a distal direction, a capability that is essential for the apical region and the apical growth.

Analyses in rust fungi and yeasts showed an inhibition of the mechanosensitive channels by gadolinium ions ( $Gd^{3+}$ ). In yeasts a function seems plausible in connection with the control of the cell cycle.

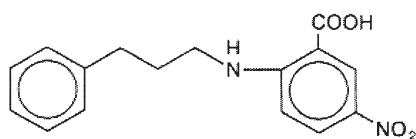
#### D. Anion Channels

The movement of cations across cell membranes must be compensated for by appropriate shifts of anions. A variety of anion channels as well as symporters and antiporters are available.

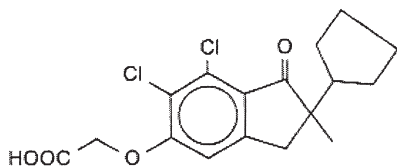
There are anion channels similar to the cation channels located in the plasma membrane and tonoplast.  $Cl^-$  channels are particularly well examined. A striking feature is the binding of auxin at the outside of the channel (121). Inhibition of the chloride influx is a direct consequence. Thus, the activation potential of the anion channels becomes negatively shifted. In the next step the anion channels respond by opening,  $Cl^-$  ions flow out of the guard cell, and the membrane becomes depolarized. If the resting potential of the cells remains for some time in the range of the activation potential, the anion channels are closed, with a half-life of 10–12 s.  $Cl^-$  no longer flows out and membrane resistance is increased.

Subsequently another process, which is independent of auxin binding, is triggered at the membrane. The  $H^+$ -ATPase in the plasma membrane is activated. The influx of potassium to the stomatal guard cells represents the final, osmotically active step in the regulatory sequence and causes volume increase of the stomatal guard cells by passive water influx and the opening of the pore. It should be noted that potassium channels are not directly influenced by auxin. There are several possibilities for the control of anion channels as for the cation channels: voltage-sensitive, ion-sensitive (e.g.,  $Ca^{2+}$ ), and mechanosensitive channels. A summary is presented by Tyerman (103) and Hedrich and Jeromin (122).

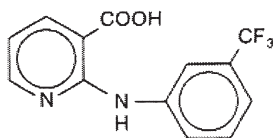
The most effective anion channel blockers are 5-nitro-2-(3-phenylpropylamine)benzoic acid (NPPB), indole acetic acid (IAA) 94, niflumic acid,



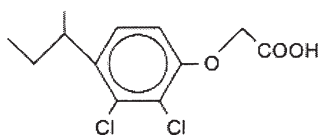
NPPB  
5-nitro-2-(3-phenylpropylamino)benzoic acid



IAA-94  
[[6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1H-5-yl)oxy]-acidic acid



niflumic acid  
2-( $\alpha, \alpha, \alpha$ -trifluoro-m-toluidine)-pyridine-3-carboxylic acid



ethacrynic acid  
[2,3-dichloro-4-(2-methylenbutyryl)-phenoxy]-acidic acid

**Figure 54** Anion channel blockers.

ethacrynic acid (Fig. 54), as well as the stilbene derivative 4,4-diisothiocyanate stilbene-2,2'-disulfonic acid (DIDS), and 4-acetamido-4'-isothiocyanostilben-2,2'-disulfonic acid (SITS), anthracene-9-carboxylate (A9C), and  $\text{Zn}^{2+}$ . The inhibition depends on the channel as well as the plant. Frequently it is less specific in comparison to different cation channel blockers. Anion channels are in some cases permeable for several anions, e.g., for  $\text{Cl}^-$ ,  $\text{NO}_3^-$ , and malate in plasma membranes of stomatal guard cells and for  $\text{Cl}^-$  and  $\text{NO}_3^-$  in tonoplasts.

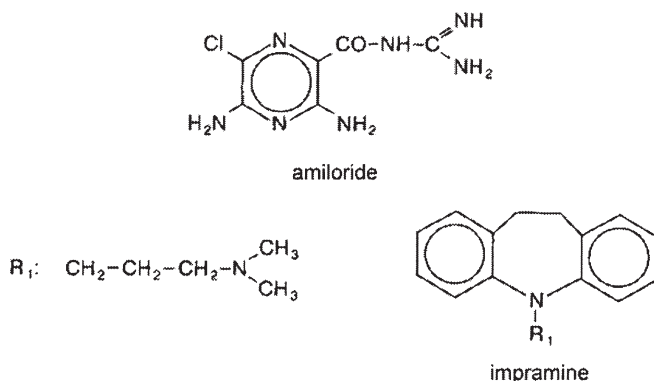
## E. Carriers

Ion channels and carriers share some common features: they permit diffusion along a gradient and show both a concentration- and a voltage-dependent saturation. Whereas an open channel allows the passage of  $>10^6$  ions  $\text{s}^{-1}$ , carriers provide a turnover rate of  $10^4$ – $10^5$  ions or molecules  $\text{s}^{-1}$ . Symport and antiport are different carrier mechanisms (cf. earlier discussion).

Carrier mechanisms have been examined particularly well in cells with high transport activities. An example is sucrose transport, which includes loading of the sieve tubes at the source as well as unloading at the sink. This long-distance transport therefore includes several carrier-mediated steps through the plasma membrane of the assimilating cells, the conducting cells of the phloem, and the storage cells. The sucrose/ $\text{H}^+$  antiport moves sucrose against a concentration gradient from the cytosol into the vacuole. The driving force is provided by the  $\text{H}^+$  efflux from the vacuole along its concentration gradient. This process is maintained by the V-ATPase in the vacuole membrane, which pumps  $\text{H}^+$  into the vacuole under ATP consumption. This explains the inhibition of sucrose import by diethylstilbestrol, *N,N'*-dicyclohexylcarbodiimide (DCCD), *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoic acid (PCMB) (cf. Table 6), which also inhibit the import to tonoplast vesicles of *Beta vulgaris* (123).

Sucrose/ $\text{H}^+$  symporters are available as carriers for the sucrose import into phloem cells. Experiments with vesicle preparations from the plasmalemma of *Beta vulgaris* leaves (124, 125) proved the dependence on the pH gradient. The import can be turned off by the protonophore carbonylcyanide-*m*-chlorophenyl hydrazone (Table 3), resembling the inhibition of the plasmalemma P-ATPase by diethylpyrocarbonate or *p*-chloromercuribenzolsulfonic acid (cf. Table 3).

Corresponding hexose transporters are found for glucose and fructose, which have been characterized in isolated plasma membrane and tonoplast vesicles (126).  $\text{H}^+$ /cation antiporters are widely distributed in the plant kingdom. A closer characterization was carried out for the  $\text{Na}^+/\text{H}^+$  antiport of *Beta vulgaris* tonoplasts where  $\text{Na}^+$  is taken up by the vacuole against a concentration gradient. This process is characteristic of halophytes and salt-tolerant glycophytes. Amiloride (Fig. 55), a diuretic pharmacoply applied in human medicine, was used as a competitive inhibitor of the  $\text{Na}^+/\text{H}^+$  antiport of vacuoles (127). Amiloride interferes with several components of the  $\text{Na}^+$  transport, i.e.,  $\text{Na}^+$  channels, symporters, and antiporters. A 170-kd polypeptide could be identified as antiporter. The  $\text{Mg}^{2+}/2\text{H}^+$  antiporter of the lactifers of *Hevea brasiliensis* is also blocked by amiloride



**Figure 55** Inhibitors of  $\text{Na}^+$  and  $\text{Mg}^{2+}/\text{H}^+$  antiports.

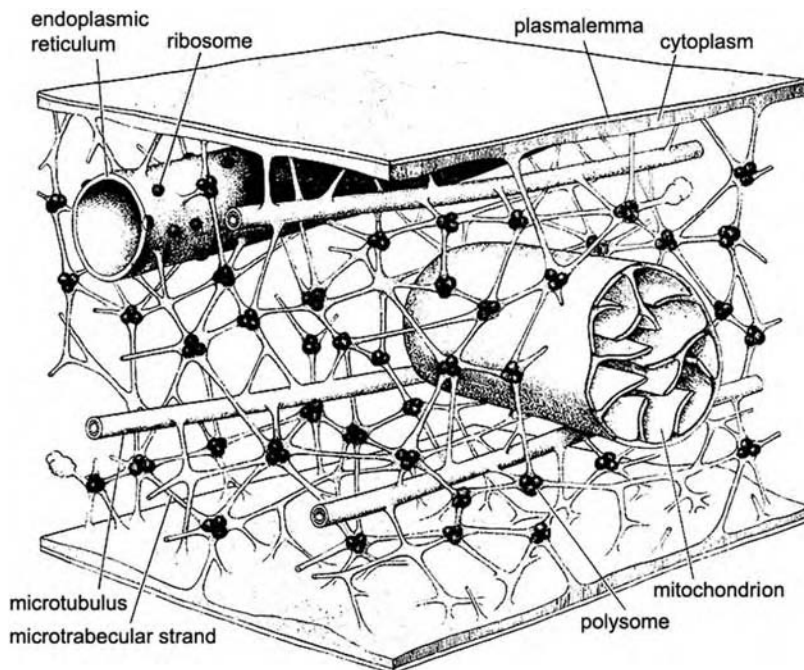
as well as by the dibenzoazepine derivative imipramine. This antiporter is responsible for the approximately 10-fold enrichment of  $\text{Mg}^{2+}$  in the luteoids, lysosomal compartments in the latex (128).

$\text{K}^+$  transport across the plasmalemma and tonoplast membrane is not restricted exclusively to ion channels; it frequently depends on  $\text{K}^+/\text{H}^+$  antiporters. It is assumed that the effect of syringomycine (cf. Section Host pathogen relations: II. Bacteria, fungi) depends not only on the stimulation of the plasmalemma ATPase, but also on a putative attack on the  $\text{K}^+/\text{H}^+$  antiporter, which triggers a  $\text{K}^+$  efflux from the cell, a typical event in many plant diseases and hypersensitivity reactions. The similarity to the effect of abscisic acid, which also evokes a  $\text{K}^+$  efflux from stomata, is apparent.

## XV. ATTACK ON THE CYTOSKELETON

The cytoplasm shows a highly organized structure, which is essentially determined by the cytoskeleton. If cells are extracted by nonionic detergents such as Triton X-100, which remove membranes and less stable cytoplasmic components, then filamentous structures that are subsumed under the term *cytoskeleton* remain. The main components in plants and fungi are microfilaments and the microtubule.

This network of more or less rigid components forms a very dynamic and flexible system, which allows fast changes of the three-dimensional organization of the protoplasts. This is an important prerequisite for motility experienced in protoplasmic streaming, active movement of cell organelles, flagellar movement, or cell division. It is assumed that the



**Figure 56** Model of the microtrabecular net linked to the cytoskeleton and different cell organelles. (From Ref. 130.)

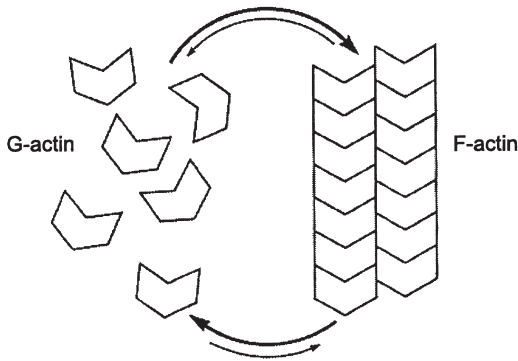
integration and control of these different fiber systems are provided by an additional metastable network of proteins, the microtrabecular network (129) (Fig. 56). It is linked with the cytoskeleton to a network, which is probably lost by detergent extraction.

### A. Inhibition of Microfilament Functions

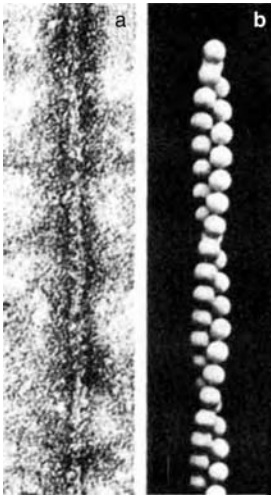
Actin is one of the most abundant proteins of eukaryotic cells. It represents the main component of the microfilament system. Actin consists of a polypeptide chain of 375 amino acid residues and binds ATP and ADP with high affinity. Adenosine triphosphate is hydrolyzed to ADP and phosphate by both filamentous polymer actin (F-actin) and globular monomeric actin (G-actin).

The physiological function of actin largely depends on its capacity for polymerization and filament formation (Fig. 57). The F-actin filament is a double-stranded, right-handed helix with 14 actin molecules per





**Figure 57** Polymerization and depolymerization of actin. (From Ref. 131.)



**Figure 58** Actin filaments. a, electron microscopic photograph of *Acanthamoeba* sp.; b, model of an actin filament. (From Ref. 132.)

turn in each strand. The pitch is 72 nm, the diameter approximately 6 nm (Fig. 58). Actin filaments are polar structures with two different ends. Actin polymerizes more quickly at the pointed than at the indented end of the filaments (cf. Fig. 58). The distribution of G- and F-actins in the plant cell is regulated by actin itself, by hydrolysis of F-actin-bound ATP, as well as by different actin binding proteins. Changes in the average filament length cause changes in the viscoelastic properties of the cytosol.

## B. Inhibition of the Protoplasmic Streaming

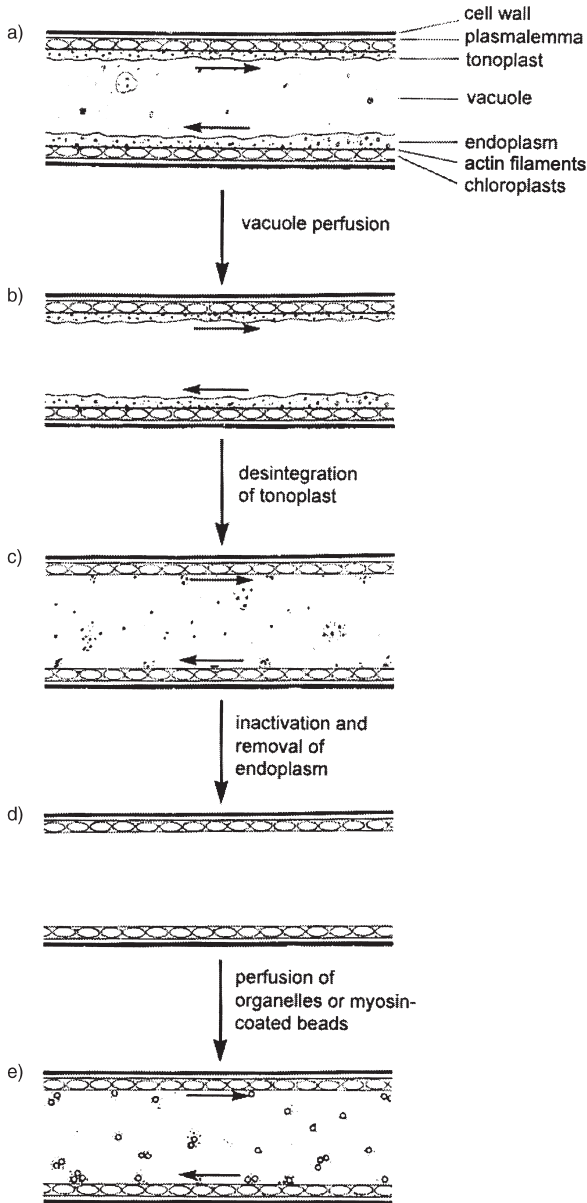
Protoplasmic streaming is an ATP-dependent, active movement of the cytoplasm. It is observed in large, mature plant cells and provides the mixture of the cell contents. The movement is driven by shear stress, which arises between a peripheral, stationary border layer and an inner mobile phase of the cytoplasm. The microfilament system, which converts chemical energy into mechanical work similarly to muscle movement by actin–myosin interaction, is responsible for this movement. The ATP is consumed; the streaming takes only place in presence of  $Mg^{2+}$  ions.

In the presence of ATP actin forms a complex with myosin, which also occurs in plants. As shown by in vitro systems, actin filaments can glide over immobilized myosin heads. The energy for this process is provided by ATP hydrolysis (133).

Major progress has been made in the analysis of the internodal giant cells of Characeae. All chloroplasts of these algae remain in this case in the stationary ectoplasm. Bundles of actin filaments are anchored at the inner surface of the chloroplasts. Myosin, however, is found in the streaming portion of the cytoplasm as protuberances on endoplasmatic organelles. The subcortical fibrils are branched to endoplasmatic filaments, which are required for the production of the shear stress in the region of the boundary layer and contain mobile, endoplasmatic myosin. Movement depends on a gliding of mobile, myosin-containing filaments along stationary actin filaments (sliding filament mechanism). The ATP-dependent force is released by myosin (134). In higher plants protoplasmic streaming also requires the presence of microfilaments.

Protoplasmic streaming was studied thoroughly in perfused internode cells (135). The two cell ends were cut off and the vacuole was perfused with a medium containing egtazic acid (EGTA) as a  $Ca^{2+}$  chelator (Fig. 59). The sudden decrease of the  $Ca^{2+}$  concentration in the vacuole leads to the disintegration of the tonoplast (tonoplast-free cell). The largest portion of the endoplasm is released. Myosin is finally inactivated in the presence of ethylenediaminetetraacetic acid (EDTA) or *N*-ethylmaleimide (NEM). Foreign cell organelles or myosin-coated beads can be introduced into this system subsequently. Then both cell ends are closed in order to prevent a passive movement of the intracellular liquid.

With this device it was possible to move cell organelles of the endoplasm of *Chara* sp. or even pollen tubes from lilies in *Nitella* sp. Furthermore, coated polystyrene beads ( $2.8\mu m$  in diameter) were moved in a similar way with myosin from skeleton muscle in the presence of ATP (136). According to the “self-induced translation model” (137) an adhesion of the myosin head causes local conformational changes in



**Figure 59** Perfusion of characean internode cells and introduction of heterologous organelles or myosin-coated beads: a, Intact cell; b, vacuole-perfused cell; c, tonoplast-free cell; d, endoplasmic-free cell; e, reconstituted movement. (From Ref. 135.)

the actin filament and generates an axial gradient of an electrostatic force. This mechanism causes the gliding of the myosin head along the actin filament.

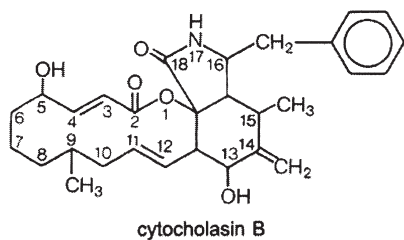
### C. Cytochalasin B

Cytochalasin B is a mycotoxin from the imperfect fungus *Helminthosporium dematioides*. It is one of the macrolide antibiotics (Fig. 60). Its structure is based on a large lactone ring, but in contrast to most other representatives of this group it lacks sugar residues. The toxin immediately and specifically inhibits the plasma streaming. Washing out can abolish this effect. Two minutes after perfusion of *Chara* sp. cells with 100  $\mu$ molar cytochalasin B the protoplasmic streaming is completely abandoned observed (138). After 30 min a significant decrease of subcortical actin filaments is observed (Fig. 61).

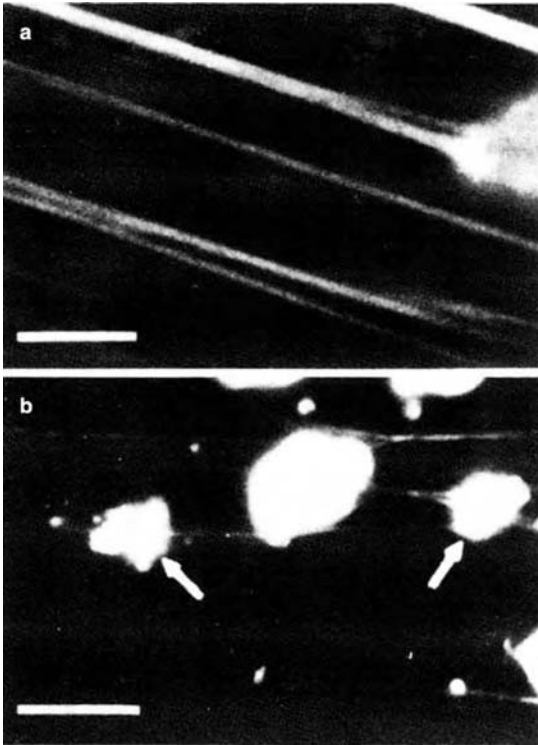
Cytochalasin B blocks the indented ends of F-actin (Fig. 57) and therefore changes the polymerization rates. The free actin subunits are then competitively inhibiting the force-generating interaction between the endoplasmatic myosin and the actin filaments. Free subunits cannot generate force together with myosin. Only extended presence of cytochalasin B finally leads to a reduced bundle number, long after the inhibition of the protoplasmic streaming has started.

The actin–myosin and the microtubule system are both involved (see later discussion) in the circulation flow of the hair cells of *Tradescantia* sp. anthers (139). This explains why combinations of microfilament inhibitors (e.g., cytochalasin D) and microtubule inhibitors (e.g., oryzalin) are particularly effective. The apical cells are characterized by a higher percentage of microtubules showing a reticular arrangement of the plasma strands. The older basal cells with long continuous plasma strands have a higher percentage of microfilaments.

Actin-controlled processes can therefore be identified by cytochalasin B binding to actin filaments. In addition to protoplasmic streaming the



**Figure 60** Cytochalasin B.



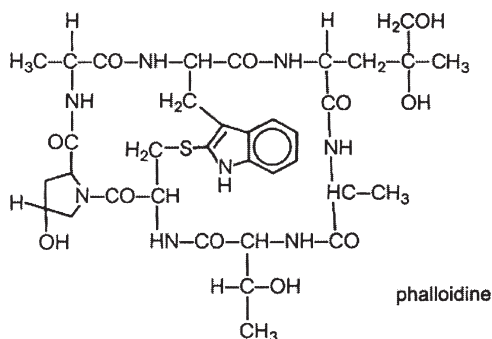
**Figure 61** Influence of cytochalasin B on the subcortical actin bundle of *Chara* sp.: a, Control with partly aggregated bundles; b, 30 min after perfusion with 100  $\mu$ molar cytochalasin B. The arrows point at aggregated material. Fluorescence photographs after treatment with the FITC-labeled actin marker “heavy meromyosin.” Windows were shot before into the dense chloroplast layer by laser light to allow visualization. Measuring bar, 10  $\mu$ m. FITC, fluorescein isothiocyanate. (From Ref. 138.)

directed movement of organelles is another example for actin-controlled processes, e.g., the transport of dictyosomes to the plasmalemma.

High cytoplasmic  $\text{Ca}^{2+}$  concentrations as well as NEM and heat (50°C) inhibit protoplasmic streaming in higher plants. The myosin function is presumably impaired in this case.

#### D. Phallotoxins

The phallotoxins phalloidine and phallacidine are among the main poisons of death caps (e.g., *Amanita phalloides*) along with amanitine. These are cyclic oligopeptides (Fig. 62). The effects are explained by using phalloidine

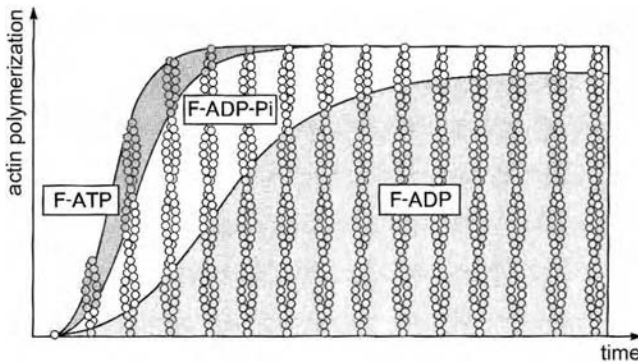


**Figure 62** Phalloidine.

as an example. This bicyclic heptapeptide with tryptathionine as the chromophore system (UV absorption at 290 nm) binds to actin molecules in actin filaments, accelerates actin polymerization, and stabilizes the filaments under conditions in which depolymerization usually takes place, e.g., the presence of DNase I, cytochalasins, 0.6 molar KJ, OsO<sub>4</sub>, ultrasound or high temperatures. Unlike cytochalasin B, the phalloidins do not permeate membranes, they must be introduced to the cell by microinjection.

Details of the polymerization process of F-actin are necessary for understanding of the phalloidine effect (140). G-actin usually contains one molecule of tightly bound ATP. Figure 63 shows the events that lead to filament growth. At the beginning rapid growth takes place with terminal F-ATP subunits. Subunits of F-ADP-P<sub>i</sub> transiently accumulate at later stages. Except the terminal F-ADP-P<sub>i</sub> subunits at the indented ends, the filament consists of F-ADP subunits in the stationary phase. Obviously ATP hydrolysis regulates the polymerization kinetics: it destabilizes the actin-actin interaction in the actin-polymer and facilitates the depolymerization process. The release of phosphate (P<sub>i</sub>), which does not immediately follow ATP hydrolysis, is the elementary step that leads to the destabilization.

Phalloidine strongly delays the P<sub>i</sub> release. Thus phalloidine extends the lifetime of the F-actin-ADP-P<sub>i</sub> species and increases filament stability (141). Phalloidine induces a conformational change that leads to the closure of the nucleotide binding site. The stabilizing effect is assumed to be responsible for the large number of microfilaments that are visible by electron microscopy after phalloidine injection. Since the toxin can be coupled to fluorescein, an elegant fluorescence microscopic proof of actin filaments is available.



**Figure 63** Scheme for the development of the actin filament during the polymerization process. F-ATP subunits, dark gray; F-ADP-P<sub>i</sub> subunits, white; F-ADP subunits, gray. (From Ref. 140.)

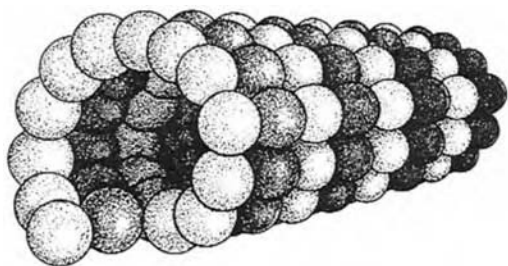
In the slime mold *Physarum polycephalum* as well as *Amoeba proteus* or fibroblast cells, microinjected phalloidine stops the protoplasmic streaming and leads to ultrastructural changes. The effects on other organisms are different. No influence on protoplasmic streaming was found, for instance, in *Vaucheria* sp. (142).

Nothnagel and coworkers (138) showed that phalloidins introduced by perfusion have no influence on the protoplasmic streaming in *Chara* sp. cells even in high concentrations. However, they accelerate the protoplasmic streaming after inhibition by cytochalasin B. The authors assume that the molecular mechanisms of streaming are different in different groups of organisms such as algae and amoebae/slime molds and that phalloidine blocks protoplasmic streaming only in those cases in which the cyclic polymerization and depolymerization of actin represent an integral constituent of the flow mechanism.

The anaesthetic lidocaine causes reversible inhibition of the protoplasmic streaming in *Vallisneria* sp. in the concentration range of 2–20 mM under alkaline conditions (143). This effect is not based on an inhibition of the motor mechanism or its energy supply but possibly on the Ca<sup>2+</sup> release from the vacuole.

## E. Inhibition of Microtubule Functions

The microtubules are filamentous structures. They are found in most eukaryotic cells and differ from the other polymer structures, the microfilaments. Unlike microfilaments the subunits are ordered in a thin hollow cylinder with a diameter of approximately 24 nm, and their



**Figure 64** Model of microtubules.

length is variable. The building block is the protein tubulin, a dimer consisting of  $\alpha$ - and  $\beta$ -tubulin. Small amounts of microtubule-associated proteins are also included. Figure 64 shows a microtubule model. As a rule, 13 tubulin units can be recognized in the cross section of the rigid structure.

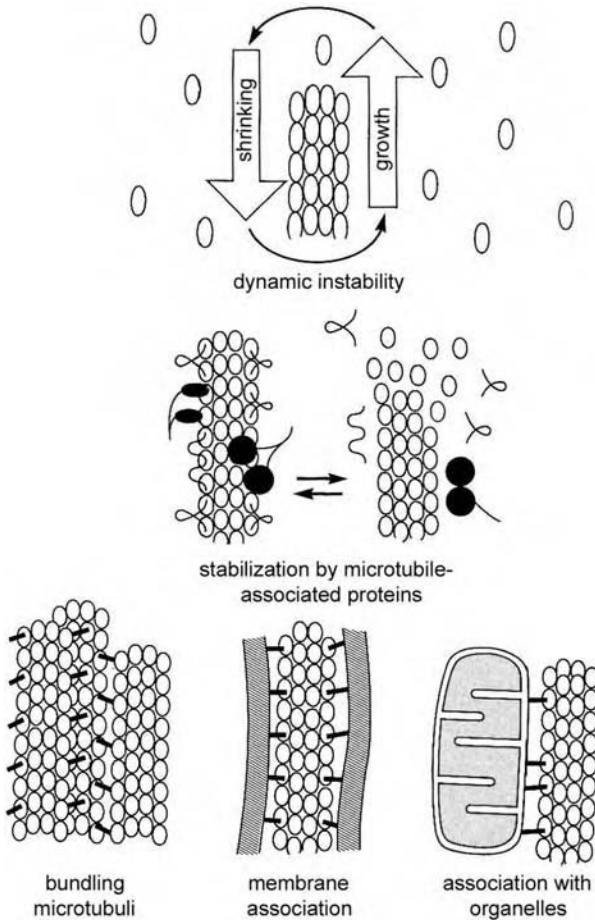
Microtubules play a pivotal role in many crucial cell functions, among them mitosis, organization, and orientation of cell organelles; intracellular vesicle transport; determination of cell form; as well as motility (e.g., flagellar motility of eukaryotes). One of the most apparent qualities of every microtubule (Mt) with the exception of the stable microtubules of flagella and cilia is their dynamic behavior. A quick exchange of the subunits between the polymers and a soluble tubulin pool is observed. This exchange plays an important role, for instance, in cell division. But even if no rearrangement of the microtubules takes place, microtubules behave dynamically. The half-life of most microtubules is in the range of a few minutes. Details are described by Gelfand and Bershadsky (144).

Buildup and destruction of microtubules are very complex events that depend on  $Mg^{2+}$ ,  $Ca^{2+}$ , calmoduline, and GTP. Increased pressure, low temperature, and  $D_2O$  destroy the microtubuli. The dynamic behavior of the microtubules is regulated on three levels:

1. The dynamic instability of the tubulin polymers (fluctuation of microtubule length by an integrated mechanism of the dynamic instability)
2. The modification of the dynamics by interacting proteins (temporary suppression of microtubule dynamics by interaction with microtubule-associated proteins; release of these proteins by phosphorylation or abolition of this suppression by ATP)
3. The formation of associations at a higher level, such as microtubule bundles or microtubule–organelle associations.

Figure 65 gives an overview.

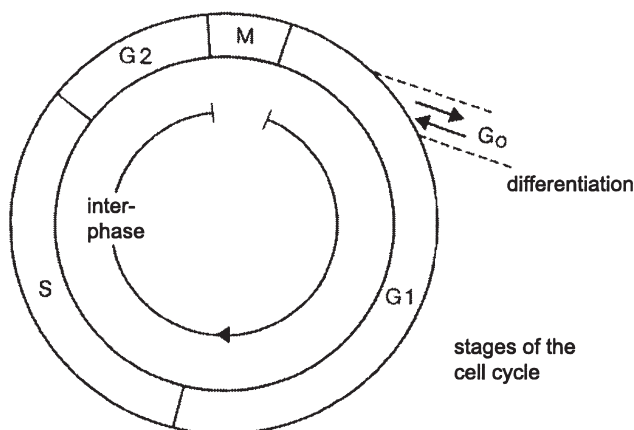




**Figure 65** Regulation of the dynamic behavior of microtubules. (From Ref. 144.)

## XVI. INHIBITION OF NUCLEAR AND CELL DIVISION

The cell cycle plays a central role in biology because the switch is set here for cell division or differentiation, respectively. The most conspicuous part of the cell cycle (Fig. 66) is mitosis (M), which provides an even distribution of the genetic material to the two daughter cells. This process is preceded by DNA replication (S phase). The M and S phases are separated from each other by the  $G_1$  and the  $G_2$  phase. Cells, which do not proliferate, but differentiate, are in the  $G_0$  phase. The division rate is probably set by the  $G_1$  phase, which serves for the preparation of mitosis.



**Figure 66** Cell cycle.

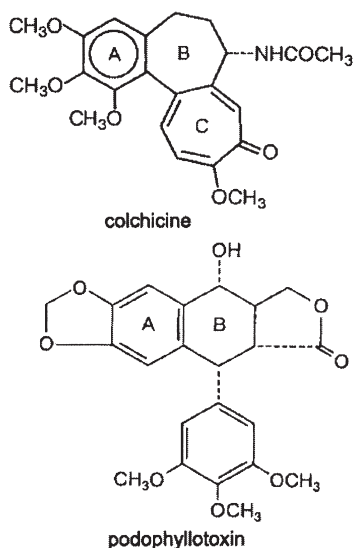
Cell division can be inhibited by blocking the cell cycle at defined stages. Inhibitors of DNA synthesis (cf. previous discussion) block the cell in the S phase. Even synchronization of the cell cycle can be achieved by reversible inhibition. In this chapter only inhibitors of mitosis are covered.

Two functionally independent cycles occur during mitosis: the chromosome cycle, which is characterized by changes in the chromatin structure (condensation and uncoiling of the chromosomes), and the spindle cycle, which is responsible for the movement of chromosomes. Many inhibitors of mitosis affect the spindle apparatus. These compounds have been examined in detail, since they are potential antitumor drugs.

The nuclear spindle is generated by spindle fibers, which consist of microtubule bundles. In principle the following mitosis inhibitors can be distinguished: (a) inhibitors that cause colchicine (C) mitosis by inhibition of the spindle formation (discussed later), (b) reagents that induce the formation of multipolar spindles, (c) reagents that cause star anaphase configurations (e.g., terbutol), and (d) inhibitors of cell plate formation.

Colchicine is the best-known spindle poison. It is the main alkaloid of the meadow saffron *Colchicum autumnale* but is also found in high concentrations in *Gloriosa superba* as well as in *Sandersonia aurantiaca*, which are found in South Africa. The condensed ring system contains one 6- and two 7-membered rings (Fig. 67).

Colchicine binds with high affinity in a 1:1 ratio to the tubulin dimer in the area of the  $\beta$ -subunit, but not to the microtubule polymer. Colchicine triggers a conformational change of the tubulins, evoking

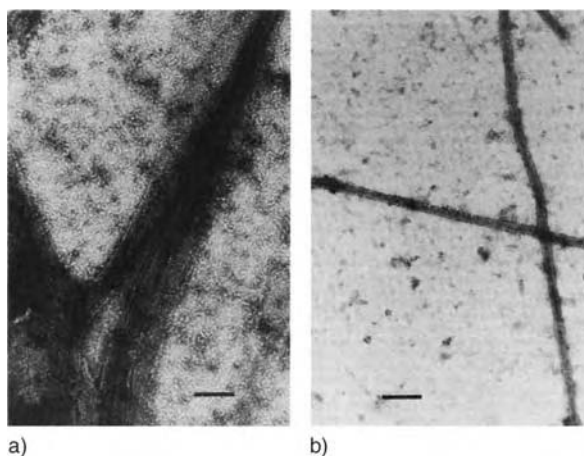


**Figure 67** Colchicine and podophyllotoxin.

a local unfolding in the carboxy terminal region of  $\beta$ -tubulins. The inhibitor blocks hydrolysis of GTP, which is bound to the  $\beta$ -subunit; thus normal polymerization cannot proceed. In addition, colchicine binding is protected by GTP.

The sensitivity of cells in higher plants is two to three orders of magnitude lower than in animal cells. Under normal polymerization conditions (presence of  $Mg^{2+}$  and GTP, absence of  $Ca^{2+}$ ) the polymerization reaction also takes place in the presence of colchicine under in vitro conditions. But because of the incorrect binding geometrical structure, large aggregates of tubulin molecules are formed, which considerably differ from microtubules with respect to their morphological characteristics (paracrystalline strands, Fig. 68), are formed.  $Ca^{2+}$  inhibits the aggregate formation. Under in vivo conditions addition of colchicine in higher concentrations causes early termination of the growing chain.

Colchicine also inhibits the formation of the spindle apparatus. During mitosis chromosomes become separated into chromatids (= daughter chromosomes), but they are no longer distributed during subsequent mitotic stages. This effect has been utilized since 1937 in breeding in order to obtain autopolyploid plants (e.g., rye or barley). The artificial polyploidization is most effectively achieved if a solution of 0.1%–0.5% colchicine is applied. Shoot or root tips are incubated for 6–24 h. An effect can, of course, be obtained only with meristematic tissue.

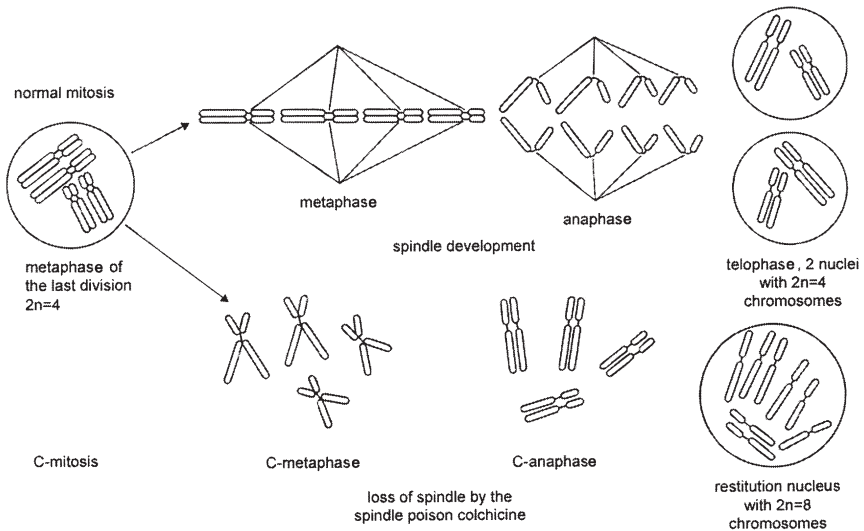


**Figure 68** Electron microscopic photographs (negative contrast method) of tubulin-colchicine complexes: a, in vitro, compared to b, tubulin complexes without colchicine. Measuring bar, 100 nm. (From Ref. 145.)

Figure 69 shows colchicine mitosis (C mitosis) compared to regular mitosis starting with a diploid set of chromosomes. Colchicine does not impair DNA replication, formation of chromatides, or the process of prophase, but only those processes that depend on the spindle apparatus (cf. Fig. 70).

The role of the microtubules is not restricted to the generation of the nuclear spindle. Microtubules also play a role in the positioning of the cell plate as well as in the orientation of the cellulose microfibrils in the growing cell wall. Thus, colchicine inhibits the formation of the cell plate and also profoundly influences the organization of the cell wall. Colchicine does not block cellulose synthesis; however, it disturbs the orientation of cellulose microfibrils. Whereas, for instance, in root cells from corn embryos parallel microfibril bundles can be recognized, the formation of these bundles is absent in the presence of colchicine. The cells show a considerable change of microfibril orientation (148). During the differentiation of vascular elements of the xylem colchicine causes wall formation smeared over the whole cell surface instead of the characteristic ring or helical wall thickening (149).

The cell diameter is kept constant at the acropetal growth of fern protonema. This process is under the control of microtubules. In the presence of colchicine, apical swelling of the protonema, which is presumably due to the destruction of microtubules (150), can be observed (Fig. 71).



**Figure 69** Schematic representation of C mitosis compared to normal mitosis. (From Ref. 146.)

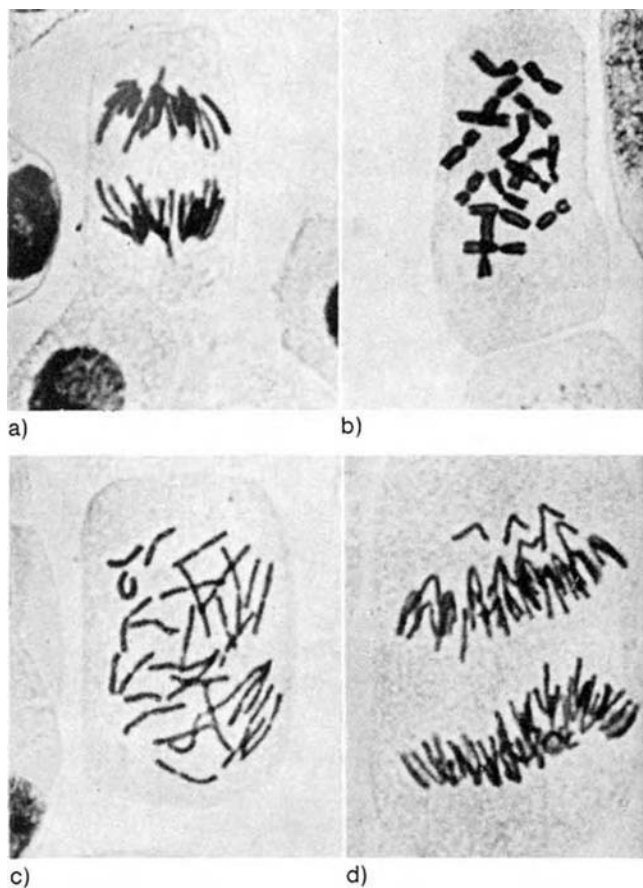
In addition to microfilaments, microtubules are involved in the protoplasmic streaming in hairs of anther filaments. The basic pattern of differentiation is not impaired by colchicine. After an injury of the central cylinder of pea roots the parenchymatic cells of the neighboring cortex tissue redifferentiate vessels and sieve elements even in presence of the inhibitor (143).

### A. Podophyllotoxin

The colchicine binding sites of tubulin also recognize podophyllotoxin (Fig. 67). Podophyllotoxin is a cell poison from roots and rhizomes of *Podophyllum peltatum* (Berberidaceae). The trimethoxyphenyl residue common to both compounds is crucial for binding, whereas the benzo-dioxol residue of podophyllotoxin and the tropolon residue of colchicine occupy different tubulin domains.

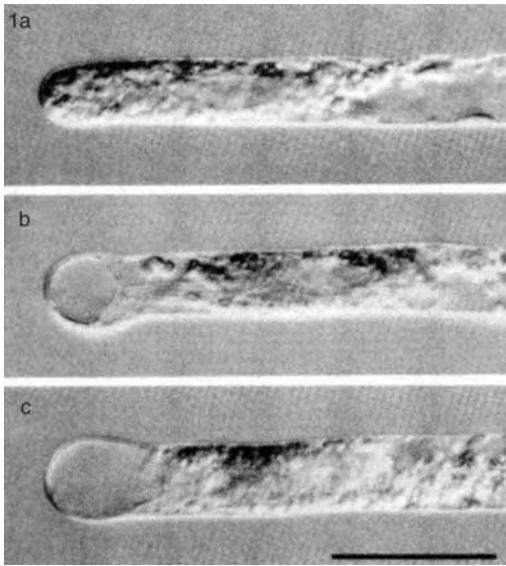
### B. Antimicrotubule Herbicides

Antimicrotubule herbicides are even more effective in plants than colchicine. They bind more specifically and in lower concentrations (micromole per liter range) to tubulin subunits and prevent polymerization. Examples of antimicrotubule herbicides are dinitroanilines such as



**Figure 70** The influence of 0.05% colchicine on root tips from onion. a, Control without colchicine (diploid anaphase); b, C-mitosis, 12 h after colchicine treatment at 24°C (extended metaphase with excessively contracted chromosomes); c, abortive anaphase from the same preparation; d, normal anaphase after removal of colchicine in a tetraploid cell. (From Ref. 147.)

trifluralin and oryzalin (cf. Section Herbicides) as well as phosphorus amides such as amiprophosmethyl (APM cf. Section Herbicides). The latter exert depolymerizing effects on existing microtubules. On the other hand, carbamate herbicides such as baran, prophan, chlorpropham, and terbutol interfere with the organization center of spindle microtubules. This effect results in the generation of multiple spindles, chromosome movement to several poles, and production of multiple nuclei (151).



**Figure 71** Influence of 5 mmol/l colchicine (b) on the growth of protonema (5 h after treatment) in comparison to the control (a). Treatment with 1 µg/ml aminophos-methyl (c) after 4 h. Measuring-bar, 50 µm. (From Ref. 150.)

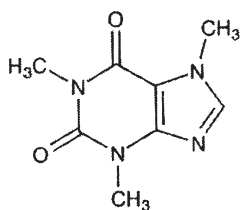
Adenosine monophosphate and pronamide are particularly effective for doubling the chromosomes, e.g., in haploid cells. The formation of numerous micronuclei containing one or few chromosomes can be accomplished by AMP treatment, followed by cytochalasin B (152). Such constructs are interesting for generating somatic hybrids.

### C. Caffeine

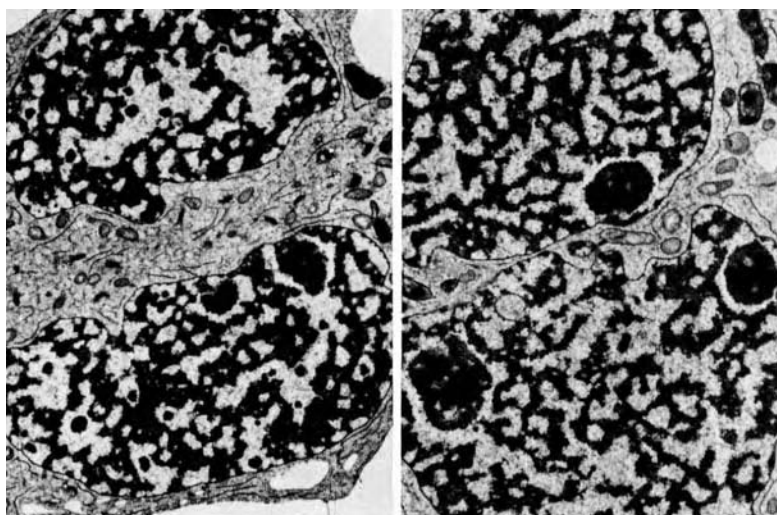
The trimethyl xanthine caffeine (Fig. 72) is an alkaloid from coffee seed ("beans"), tea, and maté leaves. In contrast to colchicine, it blocks the formation of the cell plate, which takes place in the telophase by vesicle fusion in the equator of the phragmoplast. Thus bi- and multinuclear cells are generated that at most are equipped with cell wall fragments (153).

Figure 73 shows the destruction of the cell plate in the presence of caffeine in hairs of anther filaments of *Tradescantia* sp. (154). During cytokinesis of stomatal guard cell mother cells of primary leaves in *Zea mays* (155) the inhibitor causes the development of aberrant stomata consisting of binuclear single cells. Despite the lack of a ventral wall, a pore is formed.





**Figure 72** Caffeine (1,3,7-trimethyl xanthine).



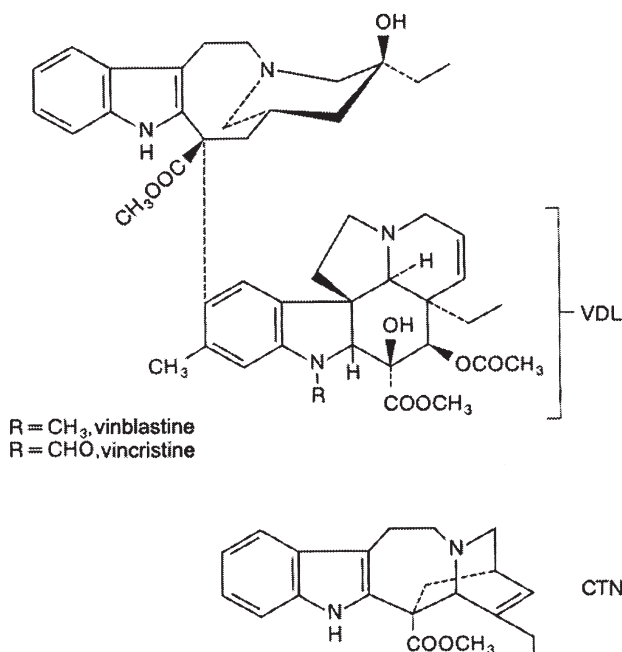
**Figure 73** Destruction of the cell plate in the presence of 5 mmol/l caffeine in hairs of anther filaments of *Tradescantia* sp. Measuring bar, 1  $\mu\text{m}$ . (From Ref. 154.)

In low concentrations caffeine does not impair microtubule function. The interference with membrane recognition or fusion is assumed to depend on disturbances of the calcium gradient, which is required for the formation of the cell plate and its fusion with the mother cell wall.

#### D. Vinca Alkaloids

A number of further highly effective mitotic inhibitors, which are used in microtubule research as well as in tumor therapy, are isolated from the leaves of periwinkle (*Catharanthus roseus*, syn. *Vinca rosea*). This member of the Apocynaceae is endemic in Madagascar. The inhibitors are indole alkaloids, e.g., vinblastine, vincristine, and vinleurosine (Fig. 74).





**Figure 74** Vinca alkaloids vinblastine and vincristine: I, VDL, vindoline; II, CTN, catharanthine.

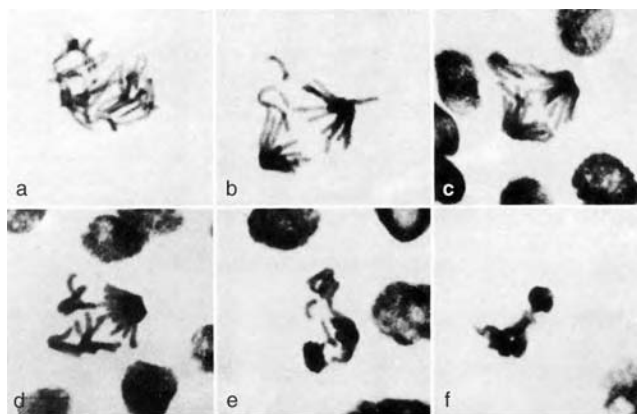
These molecules are dimers with two domains: vindoline (Fig. 74, VDL) and a restructured catharanthine (in the upper half of formulas I and II).

In contrast to colchicine, the vinca alkaloids bind to the  $\alpha$ -tubulin. In vitro, they induce tubulin to associate to linear polymers. In vivo this process leads to the generation of paracrystals. The self-assembly of tubulin to microtubules is prevented and existing microtubules disintegrate. The stable microtubules of eukaryotic flagella are hardly affected.

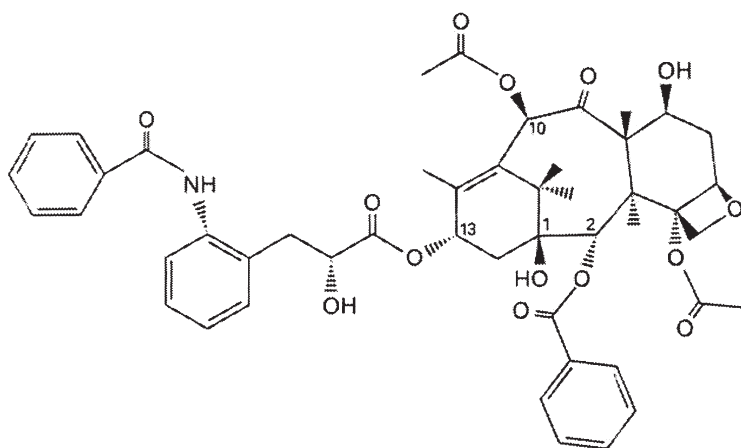
Figure 75 shows different vinblastine effects in *Vicia faba* (156). C mitoses (cf. above), chromosome losses in the anaphase, and three- and multipole anaphases, which lead to multinuclear cells with an uneven chromatin distribution, as well as agglutinated chromosomes, are examples.

Mitosis is stopped by extended incubation. Filament bundles are formed in the cytoplasm and karyoplasm, which probably do not consist of tubulin. Finally, the plasma membranes are damaged. The sensitivity to vinblastine varies in different plants. Interestingly, *Vinca rosea* is not even impaired at concentrations of  $10^{-2}$  molar vinblastine.

Taxol is a low-molecular-weight microcyclic alkaloid (Fig. 76) from the bark of the yew *Taxus brevifolia* and related species. It has been



**Figure 75** The influence of vinblastine ( $10^{-3}$  molar) on the mitosis of *Vicia faba* root tips: a, C-metaphases; b, loss of chromosomes in the anaphase; c, tripolar anaphase; d, multipolar anaphase; e, tripolar anaphase with chromosome loss; f, agglutinated chromosomes. (From Ref. 156.)



**Figure 76** Taxol.

produced recently by total synthesis. Taxol accelerates the polymerization reaction of tubulins to microtubules by lowering the critical initial concentration for nucleation. Furthermore, taxol stabilizes microtubules against depolymerization reactions, which are caused by high  $\text{Ca}^{2+}$  concentrations, lack of GTP or microtubule-associated proteins, as well as low temperatures. Analyses of *Haemanthus* endosperm (157) showed a deceleration of mitosis, formation of numerous new microtubules, as well as

increased lateral associations within microtubules and between spindle fibers. Thus the spindle is clearly reorganized, particularly in the pole areas. Chromosomal movements under the influence of taxol are characterized by a transport of chromosomes and fragments in the wrong direction (i.e., away from the polar regions) during anaphase. This suggests that extending microtubules exert a force on the chromosomes that contributes to the chromosomal movement during normal mitosis. Lateral interactions among the microtubules of spindle fibers are supported by microtubular motor proteins.

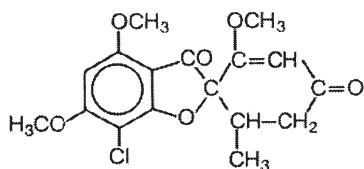
Taxol is particularly effective against oomycetes, e.g., *Phytophthora* and *Aphanomyces* spp. (158). Investigations with taxol analogs from the group of baccatins with truncated side chains at the C<sub>13</sub>-position revealed the significance of this residue for taxol activity. In higher plants taxol causes enhanced vesicle accumulation during cell division within the phragmoblast. Simultaneously it inhibits the centrifugal growth of the phragmoblast, which leads to a delay of the cross-wall formation (159). Griseofulvin, a spirobenzofurane derivative (Fig. 77), is obtained from *Penicillium griseofulvum* and is known for its fungistatic effect.

In addition to growth of fungi containing chitin in their cell walls, seed germination and root growth of several plants are impaired. Although a number of various effects, which extend from inhibition of the cell wall synthesis in fungi up to mutagenicity, are known, the crucial target is presumably the tubulin dimer, to which it specifically and stoichiometrically binds (160). Thus, griseofulvin destroys the spindle apparatus. It prevents the polymerization reaction in vitro and can even be used for the purification of tubulin.

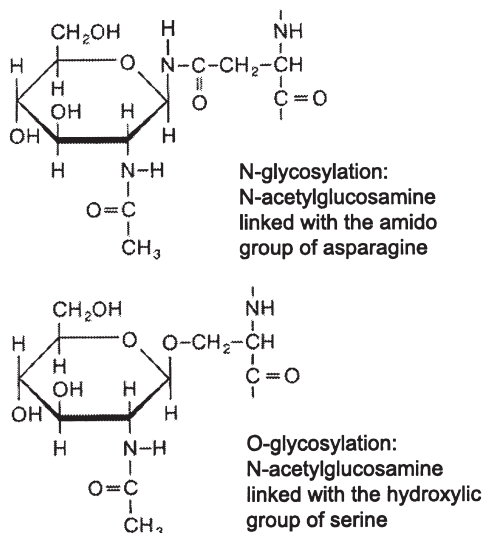
## **XVII. ENDOPLASMATIC RETICULUM AND GOLGI APPARATUS**

### **A. Glycosylation of Proteins**

Two classes of glycoproteins appear in the plant cell: membrane-associated and secreted glycoproteins. The backbone of these macromolecules consists of a polypeptide chain, which is synthesized at the rough endoplasmatic reticulum (rER). *N*-glycosylation is carried out at glycoproteins with an *N*-glycosidic linkage (between *N*-acetylglucosamine and asparagine residue, Fig. 78) in the ER lumen while the nascent polypeptide chain crosses the ER membrane (Fig. 79). An oligosaccharide block is transferred from an activated lipid carrier (dolicholphosphate) to an asparagine residue of the growing polypeptide chain. Completion of these



**Figure 77** Griseofulvin.



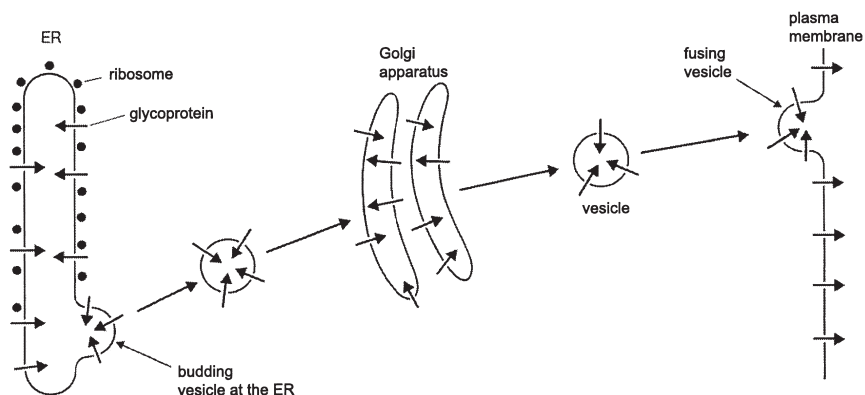
**Figure 78** Glycoprotein classes.

glycoproteins (terminal glycosylation) is carried out in the Golgi apparatus. From here the compounds are transported to their final sites of destination (Fig. 79).

A less common type of glycoprotein is characterized by an *O*-glycosidic linkage between an *N*-acetyl glucosamine and a serine or threonine residue in the polypeptide chain (Fig. 78). Primary and terminal glycosylation is exclusively carried out in the Golgi apparatus.

## B. Tunicamycin

Tunicamycin is an inhibitor that specifically prevents the synthesis of *N*-glycosylated glycoproteins. The antibiotic was isolated in 1971 from *Streptomyces lysosuperificus*. It is a hydrophobic analog of uridine diphosphate- (UDP)-*N*-acetylglucosamine and contains uracil,



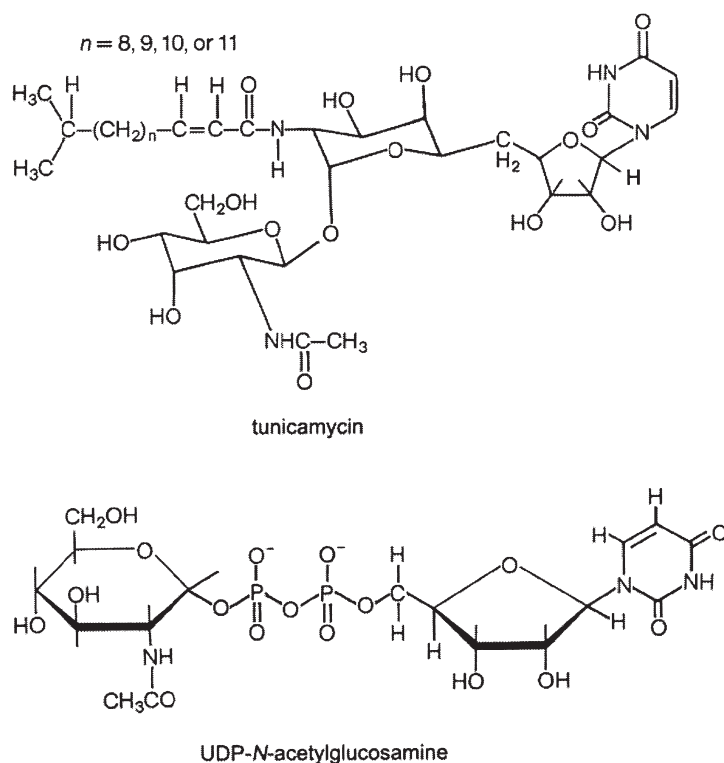
**Figure 79** Synthesis and transport of glycoproteins. ER, endoplasmic reticulum. (From Ref. 161.)

*N*-acetylglucosamine (GlcNAc), the complex  $C_{11}$  amino sugar tunicamine, and nonsaturated fatty acids containing 13 to 17 C atoms (Fig. 80).

In eukaryotic systems tunicamycin blocks the first step in the biosynthesis of *N*-glycosylated glycoproteins (Fig. 81), i.e., the transfer of *N*-acetylglucosamine-1-phosphate, which is a polyisoprenoide with 16–18 isoprene units. Obviously tunicamycin binds irreversibly to the transferase involved and inactivates the enzyme. On the other hand, it does not impair the transfer of the second Glc-Nac group to dolichol-PP-GlcNAc or terminal GlcNAc residues to the protein. Thus tunicamycin prevents the production of lipid-coupled glycoproteins whose biosynthesis involves this intermediate. The synthesis of the protein is selectively inhibited at high antibiotic concentrations, suggesting the coupling of glycosylation and protein synthesis at glycoproteins.

Several glycoproteins are found as membrane components in the plasmalemma, in lysosomes and glyoxysomes. In these cases *N*-glycosylation is specifically inhibited by tunicamycin. Valuable clues can be obtained for the membrane flow from the ER to other compartments, especially as it has to be assumed that all membrane-enclosed cell organelles contain glycoproteins. Furthermore, it will have to be clarified whether *N*-glycosylations are confined to the ER lumen.

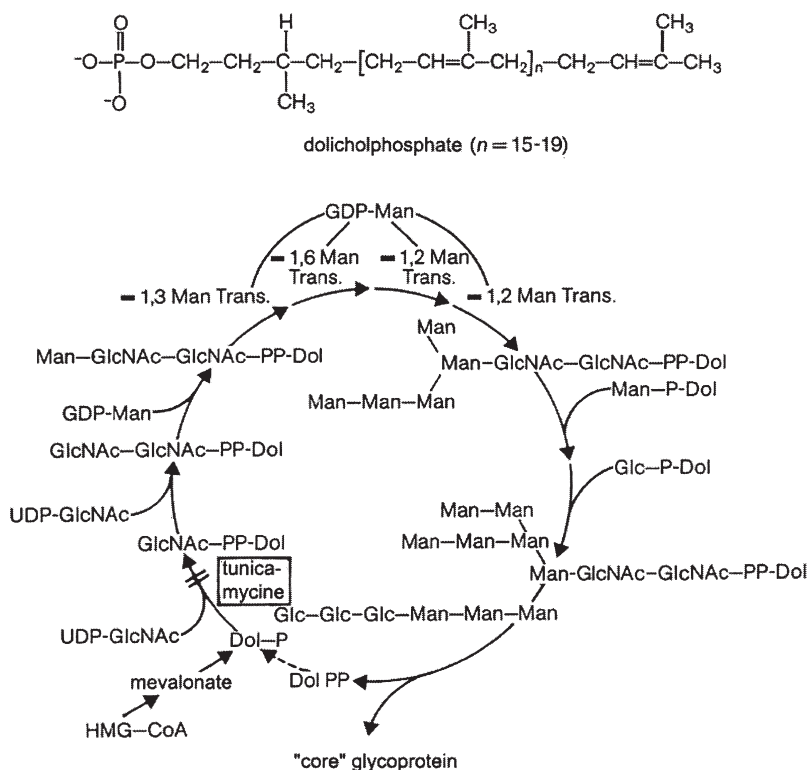
Functions of the carbohydrate moiety of membrane glycoproteins may be to control the correct orientation of the glycoproteins in the membrane and to maintain the asymmetry of the biomembrane. Membrane glycoproteins play a special role in recognition processes. These compounds are ideally suited for recognition reactions, because of the enormous variation potential of the carbohydrate moiety. As an example the



**Figure 80** Tunicamycin as an analog of uridine diphosphate- (UDP)-*N*-acetylglucosamine.

agglutination and fusion of *Chlamydomonas* sp. gametes initiate the sexual process. With the aid of tunicamycin it has been shown (163) that an antibiotic-sensitive glycoprotein is located on the flagellar surface of (+)-gametes. This glycoprotein is responsible for the agglutination to (-)-flagella, and it initiates the removal of the cell walls. A second tunicamycin-sensitive component is found on the surface of (-)-gametes and is also required for the fusion with (+)-gametes.

It has been proved for suspension cultures of soy bean cells (164) that tunicamycin also blocks the incorporation of mannose into cell wall components. However, the integration of mannose is not completely prevented since mannose is also incorporated in a process independent of the lipid-coupled saccharide biosynthesis pathway into cell wall polysaccharides. The production of certain cationic peroxidases is blocked in cell suspension cultures of *Daucus carota* in the presence of

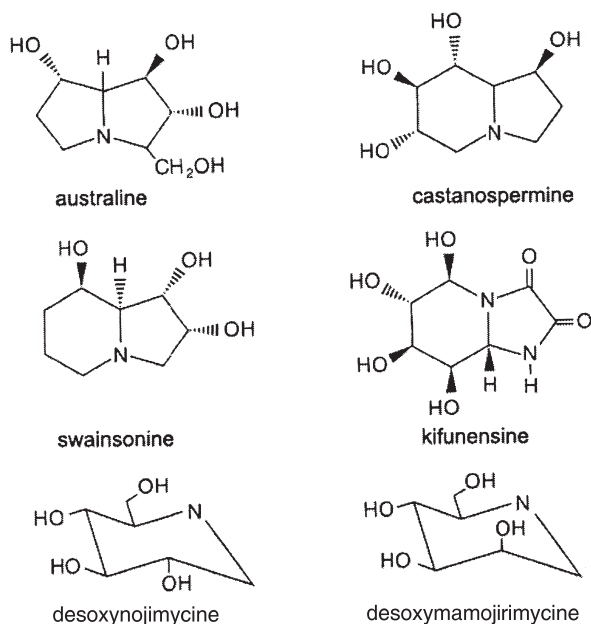


**Figure 81** Biosynthesis of glycoproteins with *N*-glycosidic bonds. Tunicamycin blocks the initial reaction (transfer of GlcNAc-1-P on dolichol-P). GDP, guanosine diphosphate; UDP, uridine diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A. (From Ref. 162.)

tunicamycin. This process is accompanied by an inhibition of the somatic embryogenesis, which can be neutralized by adding cationic horseradish peroxidase (165).

### C. Further Inhibitors of Glycosylation

Further antibiotics show chemical and biological similarities to tunicamycin: mycosporidin, streptovirudin, the antibiotics 24010 and MM-1920 (162), as well as the corynetoxins (166), which are produced during infection of *Lolium rigidum* by *Corynebacterium michiganense*. They also interfere with the formation of GlcNAc-PP-Dol.



**Figure 82** Glucosidase inhibitors.

On the other hand, bacitracine, a polypeptide antibiotic with a thiazolidine ring from *Bacillus licheniformis*, blocks not only glycoprotein synthesis in plants and fungi, but also reactions that lead from mevalonic acid to polyprenol biosynthesis by binding to farnesyl pyrophosphate. Further modification by glycosidases is also prevented by the indolizidine alkaloids swainsonine and castanospermine (Fig. 82).

Australine (Fig. 82) is a tetrahydroxylated pyrrolizidine alkaloid from the Australian tree *Castanospermum australe*. It competitively inhibits the  $\alpha$ -glucosidase amyloglucosidase, but not  $\beta$ -glucosidase,  $\alpha$ - and  $\beta$ -mannosidase or  $\alpha$ - and  $\beta$ -galactosidase (167). Furthermore, australine inhibits the glycoprotein processing enzyme glycosidase I, thus leading to the accumulation of glycoproteins with Glc<sub>3</sub>Man<sub>7-9</sub> (GlcNAc)<sub>2</sub>-oligosaccharides. Important glucosidase inhibitors (cf. Fig. 82) are the indolizidine alkaloids castanospermine from seeds of *Castanospermum australe* (inhibits the glucosidase I and II), 6-epicastanospermine, and swainsonine from the Australian plant *Swainsonia canescens* (inhibits the Golgi mannosidase II). Desoxymannojirimycine is a synthetic Golgi mannosidase-I inhibitor, desoxynojimycine from the mulberry tree *Morus* sp., an  $\alpha$ -glucosidase inhibitor. Kifunensine from the actinomycete *Kitasatosporia kifunense* is a cyclic oxamide derivative of 1-aminomannojirimycine.



It is a potent inhibitor of the plant mannosidase-I, a glycoprotein-processing enzyme (168).

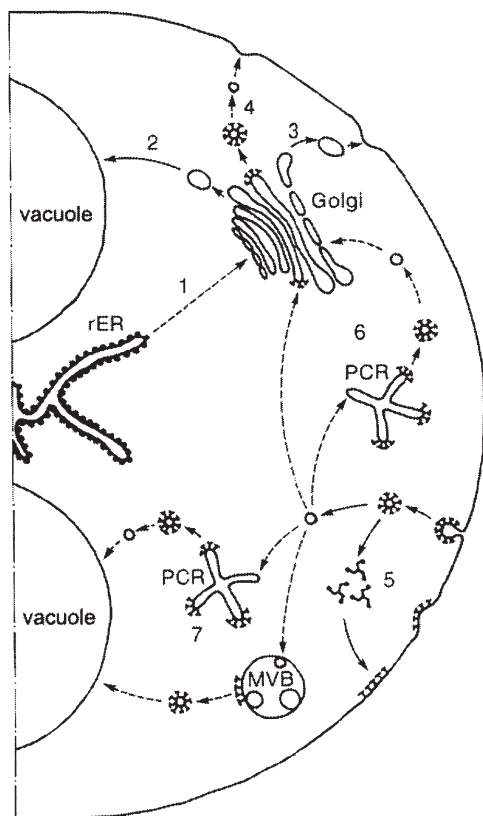
### **XVIII. COATED VESICLES AND GOLGI APPARATUS**

An intensive membrane flow takes place within the plant cell. Figure 83 describes the most important stages. The vesicle flow leads from the ER via the Golgi apparatus to the plasmalemma (exocytosis) as well as to the lysosomal compartment. On the other hand, components of the plasmalemma are reprocessed during endocytosis. Not only glycoproteins are completed in the Golgi apparatus of plants, but also the cell wall matrix proteins, hemicellulose, and pectin substances.

Endocytosis allows the recycling of receptors and other macromolecules. The corresponding membrane regions of the plasmalemma can be recognized as coated pits (Fig. 83.5) that after invagination are budded off as coated vesicles with a diameter of 90–120 nm. Considerably smaller vesicles that are coated with other proteins (diameter of 60–90 nm) are derived from the Golgi apparatus. The endocytotic vesicles are covered by a layer of the fibrous protein clathrin (Fig. 84), which forms three-armed molecules (triskelions), which are arranged on the vesicle surface to a polygonal net. This layer prevents illegitimate fusions with other vesicles and facilitates fusion with endosomes. Endosomes are vesicles in which ligands and receptors are sorted, partly degraded, or reused. Binding of clathrin to the vesicle requires adaptins as adaptor proteins. Just before fusion with the target vesicles (endosomes), the clathrin layer is removed under ATP consumption and in the presence of the heat shock protein hsp 70; Fig. 85 shows a model.

Exocytosis provides the active transport of cell wall precursors from the Golgi apparatus to the plasmalemma (Fig. 83.3) in growing plant cells. The main part of the secretion is performed via smooth vesicles, which are presumably transported with the assistance of the cytoskeleton.

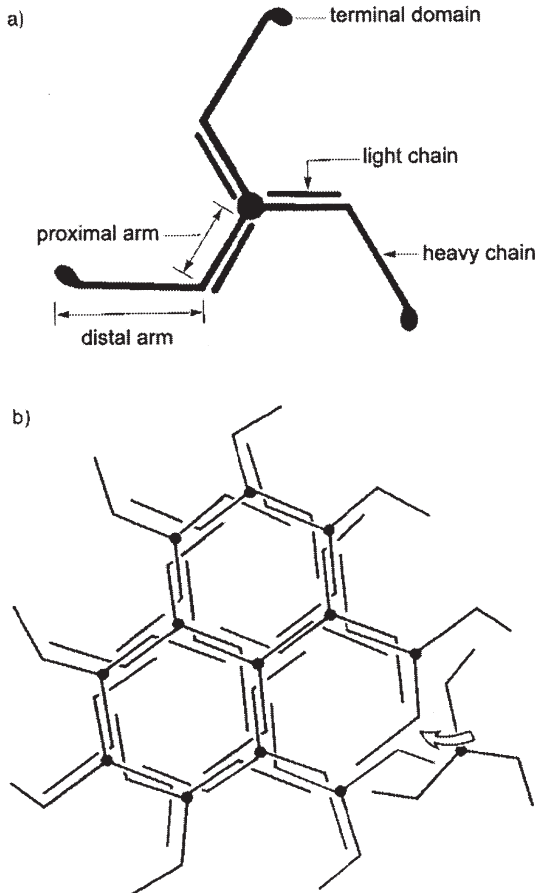
In addition, a population of coated vesicles, which are required for the transport of cell wall synthesizing enzymes from the Golgi apparatus (Fig. 83.4), is involved. One of the coat proteins is  $\beta$ -coatamer, which is related to adaptins of clathrin vesicles. Trimeric coproteins are involved in the binding of coat proteins at Golgi regions. This illustrates an intensive membrane recycling. In pollen tubes, about 80% of the membranes merging with the tip are reutilized. Endocytosis offers the possibility to control growth and development by specific removal of plasma membrane components such as ion pumps and channels or synthesis or degradation of cell wall enzymes.



**Figure 83** Vesicle transport and membrane flow in the plant cell. Open arrows: Hypothetical paths: 1, Flow of membranes and macromolecules from the rER to the cis-Golgi; 2, transportation of storage protein from trans-Golgi to the vacuole; 3, exocytosis of material in large, smooth Golgi vesicles; 4, exocytosis of material from Golgi apparatus in coated vesicle; 5, endocytosis mediated by clathrin-coated pits and vesicles; 6, transport of endocytosed material directly to the trans-Golgi via smooth endoplasmic reticulum (ER) or to the Golgi via partially coated reticulum (PCR); 7, transport of endocytosed material to the vacuole by PCR or multivesicular body (MVB). (From Ref. 169.)

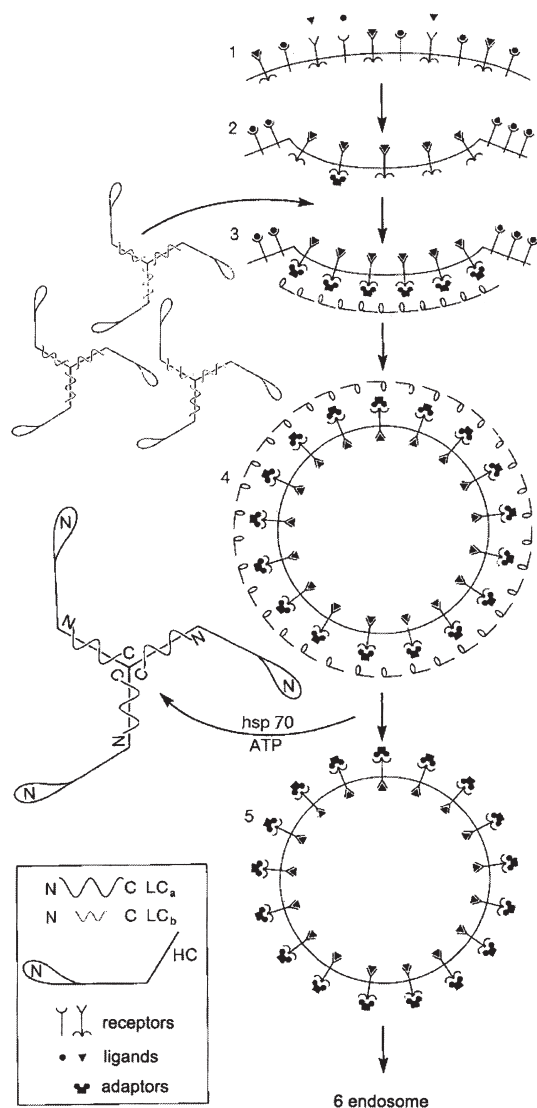
### A. Inhibitors

Brefeldin A (BFA) is a macrocyclic lactone (Fig. 86), which is synthesized from palmitate by various fungi, e.g., *Penicillium brefeldianum*, *Alternaria carthami*, the pathogen of the leaf spot symptom (cf. previous discussion). Under the influence of the toxin the membrane flow from the ER and thus

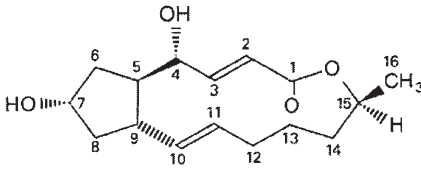


**Figure 84** Model of a clathrin triskelion: a, Clathrin structure; b, packing of triskelions to a clathrin cage. (From Ref. 169.)

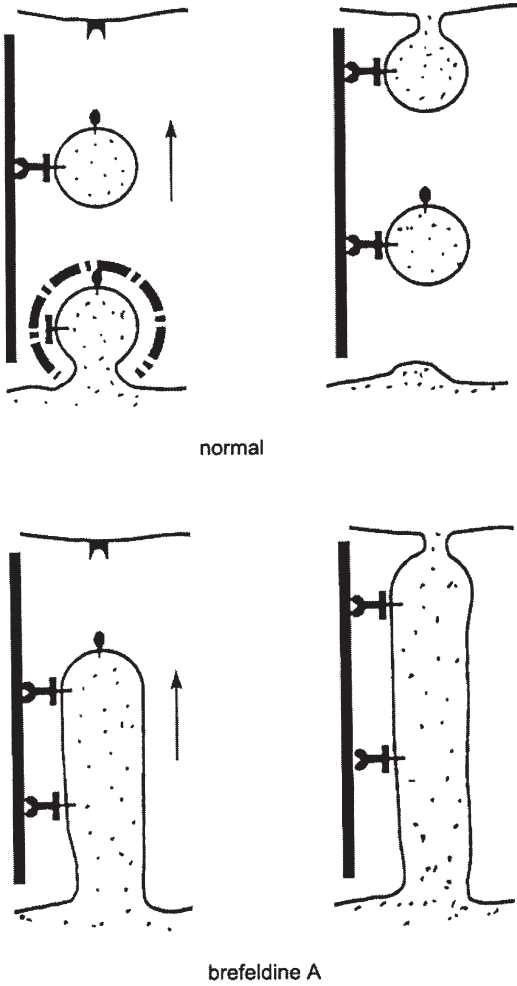
the transportation of secreted proteins to the Golgi apparatus is inhibited. In addition, disintegration of the Golgi apparatus takes place and many Golgi enzymes are redistributed to the ER. Exocytosis is stopped. A potential target of BFA is seen in the loss of certain coat proteins ( $\beta$ -COP) in Golgi membranes, which usually mark adhesion sites for the microtubules. In the presence of BFA uncoated tubes are pulled away from the membranes, which otherwise would bud vesicles. However, these tubes are nevertheless able to fuse. Therefore, Golgi cisternae are fused and tubular contacts are formed with the ER in the presence of BFA; Fig. 87 shows a model (171).



**Figure 85** Assembly and dismantling of clathrin during receptor-mediated endocytosis: 1, Ligands bind to receptors during receptor-mediated endocytosis; 2, adaptors interact with the clathrin coat; 3, clathrin is derived from a soluble triskelion pool in the cytoplasm and assembled to polyhedron lattices; 4, adaptor-associated receptors are thereby enclosed in the coated vesicle; dismantling is presumably caused by the cytosolic heat shock protein hsp 70 in an ATP-dependent process; 5, the uncoated vesicle finally merges into an endosome. ATP, adenosine triphosphate. (From Ref. 170.)



**Figure 86** Brefeldin A.



**Figure 87** The effect of brefeldin A (BFA). (From Ref. 171.)

The transport of an auxin-binding protein from the ER to the Golgi apparatus can also be inhibited by BFA (172). Thus, the normal relocation to the plasma membrane and the cell wall, where the primary binding of auxin is usually carried out, is interrupted. Large aggregates of Golgi stacks close to the cell nucleus are formed in suspension cultures of maple cells in the presence of BFA (173). The main target is the inhibition of the transport between trans-Golgi wells and secretory vesicles. The subsequent fusion with the plasma membrane is also inhibited. Vesicles with large quantities of xyloglucan, which represents a principal component of hemicellulose, accumulate instead.

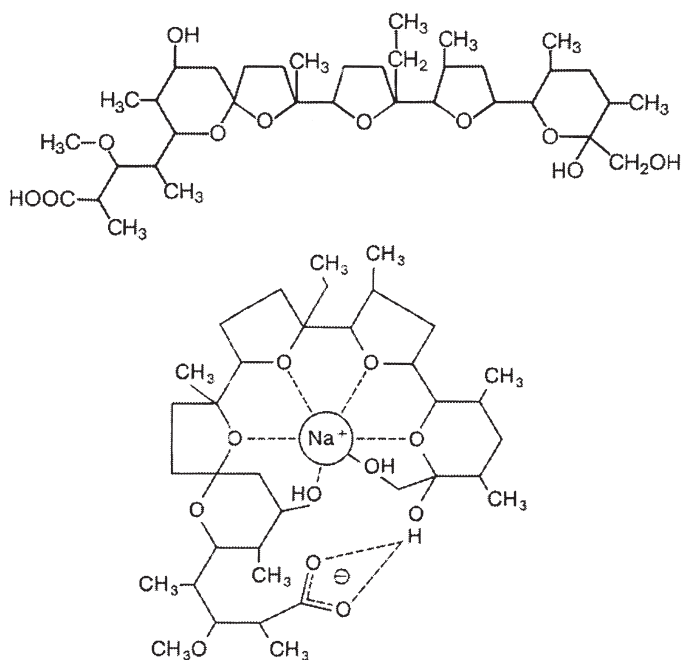
## B. Guanosine Triphosphate Analogs

Nonhydrolyzable GTP analogs such as GTP $\gamma$ S inhibit the intra-Golgi transport and the budding of secreted vesicles from the trans-Golgi network (summary by Burgoyne (174)). This indicates a participation of the trimeric G protein whose  $\alpha$ -subunit is activated by GTP $\gamma$ S. Thus uncoating is prevented and the membrane flow is stopped. AlF<sub>4</sub> mimicks this effect since it also activates the  $\alpha$ -subunit. The smaller monomeric G proteins are also involved in the membrane flow in addition to the trimeric G proteins.

Monensin, an antibiotic from *Streptomyces cinnamonensis*, is a Na<sup>+</sup> ionophor (Fig. 88), which mediates the Na<sup>+</sup>/H<sup>+</sup> exchange. The molecule complexes ions by its cyclic form, which is stabilized by hydrogen bridges between carboxyl and hydroxyl groups. The alkyl groups are directed to the outside and lead to the hydrophobic property of the complex—a prerequisite for the membrane passage. The compound was initially used as coccidiostatic antibiotic in poultry breeding. Later it was shown to be an important tool for the functional analysis of the Golgi apparatus. The affinity for K<sup>+</sup>, another competitor, is 10 times weaker than that for Na<sup>+</sup>. A summary is found at Mollenhauer (175).

Structural alterations and the destruction of dictyosomes are already observed at 10<sup>-5</sup> molar concentrations in cells of corn root tips (176). In *Euglena* sp., which has particularly large cisternae with a clear polar gradient, noticeable effects are recognized on the middle and distal cisternae (swelling after glutaraldehyde fixation) 5 min after addition of the antibiotic, whereas the proximal half remains unchanged (177). An extreme swelling was observed in thylakoids of exposed caulonema cells of *Funaria hygrometrica* (178).

Furthermore, lipids accumulate in the Golgi apparatus, and the lipid transport to the plasma membrane is consequently decreased in the presence



**Figure 88** Monensin: above, in the open form; below, in the complexed form. (From Ref. 175.)

of monensin. Phospholipids and long-chain fatty acids are particularly involved ( $>18$  C atoms) (179).

Since monensin exchanges protons for  $\text{Na}^+$  the observed effects could be explained by an osmotic mechanism. Monensin-induced swellings should therefore be characteristic of cell compartments with active proton pumps, provided that sufficient supply of monovalent cations is available (179).

The secretion of cell wall polysaccharides of the cell wall matrix via the Golgi apparatus can be blocked by KCN, azide, and other inhibitors of the energy supply (180). An enlargement of the Golgi cisternae is induced. This means that vesicle formation is energy-dependent. For suspension cell cultures from maple Zhang and associates (181) reported an accumulation of swollen vesicles, which are derived from a budding process from the trans-Golgi network and trans-Golgi cisternae in presence of monensin. Since these are extensively acidic compartments, it is obvious that the monensin-mediated exchange of protons against osmotically effective substances such as  $\text{K}^+$  and  $\text{Na}^+$  leads to swelling.

## XIX. MITOCHONDRIA

### A. Inhibition of the Respiration

The universal energy currency of the cell is ATP. It is provided by the oxidative phosphorylation in mitochondria. This process is driven by electrons, which move from reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide (FADH<sub>2</sub>) along the electron transport chain (respiratory chain in the inner mitochondria membrane) and finally reduce molecular oxygen together with H<sup>+</sup> to water. Synthesis of ATP is coupled to this electron transfer. Several antibiotics and drugs are available to block oxidative phosphorylation at different sites.

### B. Inhibition of the Electron Transport Chain

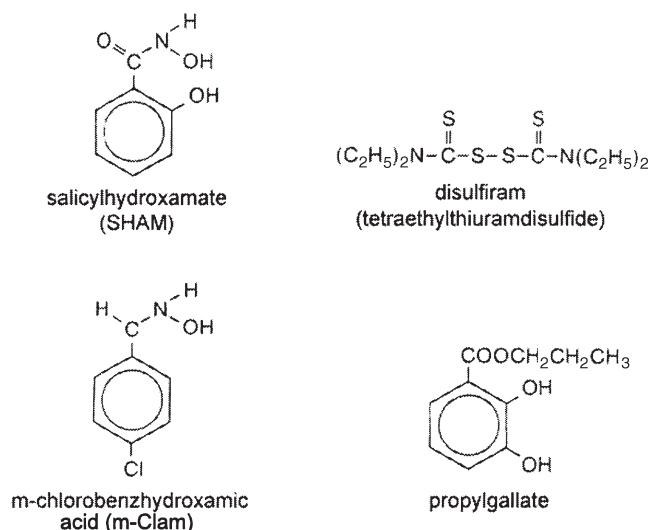
The electron transport chain of the mitochondria consists of four respiratory complexes, which represent individual mini-electron transfer chains. In addition, two mobile carriers, (Coenzyme Q [= ubiquinone] and cytochrome C), which mediate between the complexes (Fig. 89), are involved. The complexes I, III, and IV function as proton pumps.

The electron transport chain can be blocked by different inhibitors (Fig. 89) at various sites. This approach provided initial clues about the order of the electron carriers in the respiratory chain. The complete chain could finally be divided into individual segments with the aid of these inhibitors. Four inhibitors, which interfere with the iron sulfur centers of complex I and coenzyme Q, block the electron transport in the region between NADH (generated by the substrate oxidation in the matrix) and coenzyme Q (Fig. 90).

Rotenone (Fig. 90) is an extremely poisonous, complex isoflavonoide, which occurs in the Fabaceae *Derris* and *Lonchocarpus* spp. It was used as a fish poison and insecticide. The phytoalexin glyceollin (cf. Section: Host pathogen relations. II. Bacteria, fungi) has a comparable effect. Piericidin A (Fig. 90), an antibiotic from *Streptomyces mobaraensis*, resembles coenzyme Q. It is very firmly bound by mitochondria, presumably at the same site as coenzyme Q. The barbiturate amytal (Fig. 90) shows similar effects. The inhibitors mentioned do not interfere with succinate oxidation and do not block the cyanide-insensitive, NADH-dependent substrate oxidation. The coenzyme QH<sub>2</sub>-cytochrome C-reductase is inhibited by antimycin A (Fig. 90), an antibiotic from *Streptomyces griseus*. The basic structure contains a dilactone ring, which is linked to 3-formamide salicylic acid by an amide bridge. Antimycin A binds to a low-molecular-weight protein in complex III and blocks thereby the electron transfer to cytochrome c.







**Figure 91** Inhibitors of the alternative (cyanidine-resistant) oxidase.

It can be inhibited by cyanide, azide, carbon monoxide, and hydrogen sulfide. These compounds are primarily known for their extreme toxicity to humans. Figure 89 explains why several inhibitors do not impair oxygen uptake of many plants (e.g., *Arum maculatum*) despite their inhibition of the electron transport. In this case, the alternative electron transport is turned on.

The alternative electron transport chain is found in all higher plants. It is not inhibited by cyanide. It branches off from the main chain in the region of coenzyme Q (Fig. 89) and terminates with an alternative oxidase, which is located in the inner mitochondria membrane. This electron transfer can be inhibited by substituted hydroxamates (e.g., salicyl hydroxamate, m-clam), propylgallate or disulfiram (Fig. 91), free fatty acids, and thenoyltrifluoroacetone. These are compounds known as free radical scavengers (182). The ionophor valinomycin (cf. Fig. 33) is an even more potent inhibitor, e.g., in potato tubers in the presence of  $K^+$  (183). Salicylic acid increases cyanide-resistant respiration in *Nicotiana tabacum* without influencing the capacity for cytochrome c-dependent respiration (184).

The energy of the alternative electron transport cannot be used for ATP synthesis. Instead heat is liberated. This process takes place if the regular cytochrome pathway is blocked by respiratory inhibitors or saturated by an excess of reductants, e.g., NADH or NADPH. This occurs in certain situations, e.g., during seed germination, wound healing,

flowering, as well as cold exposure, in which an increased activity of the alternative oxidase is recorded. This process has been extensively studied for thermogenic species from the Araceae family (e.g., arum *Arum maculatum*, voodoo lily *Sauromatum guttatum*, and skunk cabbage *Symplocarpus foetidus*). The heat produced by the alternative pathway evaporates foul-smelling substances and attracts insects to pollination.

The cyanide-resistant alternative oxidase of *A. maculatum* was cloned and expressed in *Escherichia coli*. A heme synthesis mutation is suppressed (185). The identity of the enzyme and its cofactors (Fe, Cu, flavoprotein?) is not yet clear.

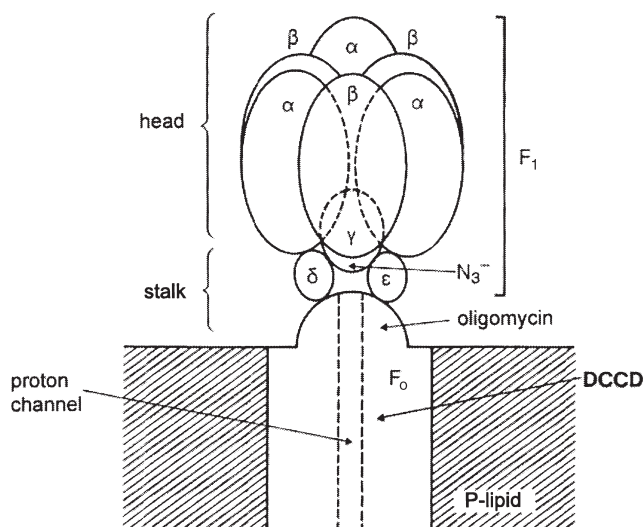
## XX. INHIBITION OF THE PROTON TRANSLOCATION AND ADENOSINE TRIPHOSPHATE SYNTHESIS

Coupling of ATP synthesis to the electron transport of the respiratory chain involves an electrochemical gradient of  $H^+$  across the inner mitochondrial membrane as predicted by the chemiosmotic theory of P. Mitchell (186). The complexes I, III, and IV of the electron transport chain serve as  $H^+$  pumps. They are incorporated into the inner membrane in such a way that  $H^+$  ions are transported from the mitochondrial matrix across the inner membrane to the intermembrane space and a  $H^+$  gradient is built up vertically to the membrane. This process represents the primary energy source for ATP synthesis.

The double lipid layer of the inner mitochondria membrane is impermeable to  $H^+$  ions. The backflow of protons takes place at defined sites, which can be recognized in the electron microscope as small spheres attached to the inner side of the membrane by stalks. It is a reversible proton-translocating ATPase, which as ATP synthase uses the electrochemical gradients of protons for ATP synthesis (or in other cases generates as ATPase such a gradient; cf. earlier discussion).

This enzyme complex belongs to the F-ATPases (=  $F_0F_1$ -ATPase) and shows a striking similarity with the F-ATPase of thylakoid membranes and the plasma membrane of eubacteria. The F-ATPase differs considerably from the P-ATPase of the plasmalemma in plants and the V-ATPase of the tonoplast. Nevertheless close phylogenetic relations exist between F- and V-ATPases (cf. above). Sequence similarities indicate a common origin from an ancestor V-ATPase. Both enzymes are not inhibited by the classic P-ATPase inhibitor, vanadate.

The name *F-ATPase* is derived from the term *factor* (from *coupling factor*). The enzyme consists of the two main components  $F_1$  and  $F_0$ , an



**Figure 92** Model of F-adenosine triphosphatase (F-ATPase). DCCD, *N,N'*-dicyclohexylcarbodiimide. (From Ref. 187.)

organization that explains the designation  $F_1/F_0$ -ATPase (cf. Fig. 92). The molecular weight of the F-ATPase is lower than that of the V-ATPase.

The F<sub>1</sub> subunit (=coupling factor 1) with the components  $\alpha$ – $\epsilon$  is visible as a small stalked circle in electron microscopic photographs. The subunit contains ATPase activity but is not able to carry out ATP synthesis separately. The stalk of the F<sub>1</sub> subunit binds to the F<sub>0</sub> subunit and has regulating functions. The hydrophobic F<sub>0</sub> subunit (*o* for *oligomycin sensitivity*) contains the proton channel. It is embedded in the inner mitochondrial membrane. F<sub>0</sub> consists of the components I–IV and includes an oligomycin binding site at the surface as well as a more deeply embedded binding site (cf. later discussion) for DCCD.

Four different inhibitor types are available for the inhibition of the ATP synthesis coupled to the electron transport: (a) inhibitors of the F-ATPase; (b) uncouplers, which prevent the utilization of the electrochemical gradient for ATP synthesis and convert the energy into heat; (c) transport antibiotics, which facilitate the uptake of ions; (d) translocase inhibitors.

### A. Inhibitors of the F-Adenosine Triphosphate

The F<sub>1</sub> subunit is modulated by several factors. It is activated by  $\text{Ca}^{2+}$ , bicarbonate, and particularly sulfite, whereas it is inhibited by

nitrate, fluoride, aluminum, and beryllium. Fluorine metal complexes  $\text{ADP}_1\text{Mg}_1\text{Al}_1\text{F}_4$  and  $\text{ADP}_1\text{Mg}_1\text{Be}_1\text{F}_x (\text{H}_2\text{O})_{4-x}$  (with  $x=1-3$ ) (188) are effective compounds. They function as phosphate analogs and form abortive complexes with ADP in the active centers of  $\text{F}_1$ . Calmoduline antagonists such as calmidazolium also show inhibiting effects, although  $\text{F}_1$  does not exhibit calmodulin dependence (189). 2-Ethoxy-carbonyl-*N*-ethoxy-1,2-dihydroquinolin (EEDQ) and nitrobenzofurazane (NbF) disable the  $\text{F}_1$  unit. The mercury reagent thimerosal (ethylmercury-thiosalicylate) is also known as a  $\text{F}_1$ -inhibitor. Azide ( $\text{N}_3$ ) blocks the  $\gamma$ -subunit of  $\text{F}_1$ .

The most frequently used inhibitors of  $\text{F}_0$  are oligomycins, macrolide antibiotics from *Streptomyces diastatochromogenes*. The multimembered lactone ring is differently substituted at the individual types. Oligomycin B (Fig. 93) is particularly effective.

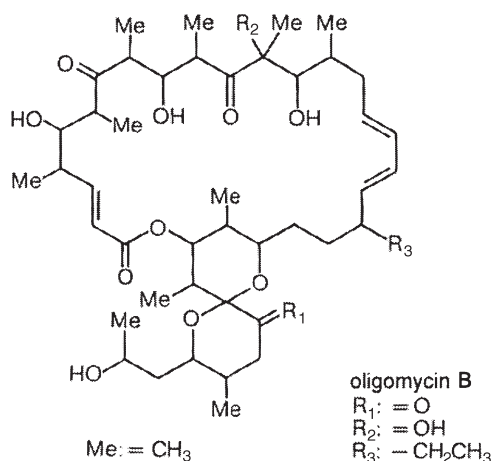
The inhibitory effect is based on the binding to an oligomycin-sensitive lipoprotein in the  $\text{F}_0$  part of the membrane-bound ATPase (Fig. 92). The antibiotic does not impair other cellular ATPases with the exception of chloroplast F-ATPase (discussed later). Rutamycin and venturicidin show similar effects.

A further inhibitor of numerous proton-translocating enzymes is the synthetic hydrophobic compound *N,N'*-dicyclohexyl carbodiimide (DCCD, Table 3), which binds covalently to the carboxylic group of an asparagine acid residue of a proteolipid in the  $\text{F}_0$  part and blocks the function of the proton channel. A single molecule per  $\text{F}_0$  is sufficient. The  $\beta$ -subunit of  $\text{F}_1$  and the chloroplast ATP synthase are blocked by DCCD (cf. earlier discussion). Furthermore, DCCD blocks the transmembrane proton translocation, which is coupled to the electron transport. Vanadate, an inhibitor of the  $\text{H}^+$ -ATPases of the plasmalemma and the soluble phosphatases, impairs neither mitochondrial nor chloroplast ATPase.

The organolead triethyllead is a potent inhibitor of F-ATPase. It is even more effective than oligomycin. The environmental relevance, however, is doubtful since concentrations in rainwater and clouds are not sufficient for inhibition (190). Accumulation is unlikely.

## B. Uncouplers

The electrochemical potential difference between the intermembrane space and the mitochondrial matrix drives the  $\text{H}^+$  movement through the F-ATPase. Abolition of the  $\text{H}^+$  gradient therefore turns off ATP synthesis without interfering with the electron transport and  $\text{O}_2$  uptake. Different uncoupling reagents (discussed later) are known.

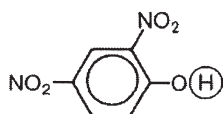


**Figure 93** Oligomycins.

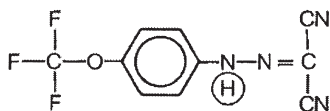
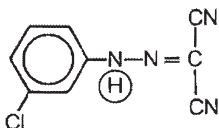
In mitochondria the electrochemical  $H^+$  gradient (proton-motive force) across the inner membrane largely depends on the electric potential gradient (in contrast to chloroplasts, in which the pH gradient has a major contribution). This is due to the poor permeability of the inner mitochondrial membrane to other cations and anions, which is different in chloroplast thylakoid membranes. Therefore, the collapse of the electric potential gradient is already sufficient in mitochondria for uncoupling. This can be easily achieved by ionophores such as valinomycin (in the presence of  $K^+$ ) (cf. earlier discussion). The membrane potential collapses because of the concomitant  $K^+$  uptake.

Permeable ions and electrogenic ionophores, which evoke a collapse of the pH gradient, are less effective in mitochondria.

Protonophores, e.g., substituted phenols such as 2,4-dinitrophenol (DNP), carbonyl cyanide-*m*-chlorophenylhydrazine (CCCP), or carbonyl cyanide-*p*-trifluoro-methoxy-phenylhydrazine (FCCP) (Fig. 94), are lipophilic, weak acids. They increase the proton permeability of membranes by moving protons in a shuttle mechanism across the membrane. The light-induced  $H^+$  uptake into the thylakoid lumen of chloroplasts is also reduced in the presence of protonophores.  $H^+$  is even released in this case. An example of the action mechanism is illustrated for DNP (Fig. 95), which is a weak lipophilic acid. It dissociates to an anion base and remains relatively soluble even without a charge-neutralizing partner. Therefore, DNP can act as a protonophore in the hydrophobic domain of the inner mitochondrial membrane.



2,4-dinitrophenol (DNP)

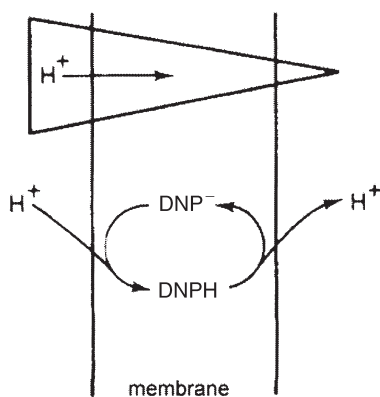
carbonylcyanide-p-trifluoro-  
methoxy-phenylhydrazone (FCCP)carbonylcyanide-m-chlorophenyl-  
hydrazone (CCCP)**Figure 94** Protonophores. Dissociating H atoms are indicated by a circle.

Those compounds that cause the collapse of the pH gradient by destroying the membrane structure (cf. Chapter 7, Herbicides) are different from the uncouplers mentioned, for instance, different lipophilic compounds such as nonionic detergents, free fatty acids (e.g., oleate, palmitate, and their significance to thermal regulation), and narcotics (e.g., chloroform, halothane). They are designated as decoupler (191). If more membrane channels are characterized in detail, further inhibitors that block mitochondrial functions will become available. The same applies to chloroplasts.

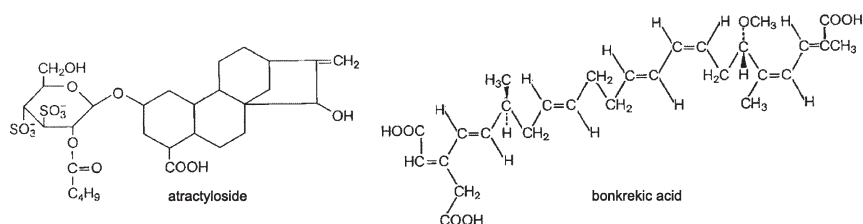
## XXI. TRANSLOCASE INHIBITORS

Neither ATP nor ADP can freely diffuse through the inner mitochondrial membrane. The transport is made possible by an ATP-ADP translocase. This specific carrier of the inner membrane couples the ADP influx into the matrix to an ATP efflux according to the antiporter principle. The proton gradient mentioned previously provides the driving force.

Atractyloside is a glycoside from the Mediterranean thistle *Atractylis gummifera* that works as a competitive inhibitor of the translocase (Fig. 96).



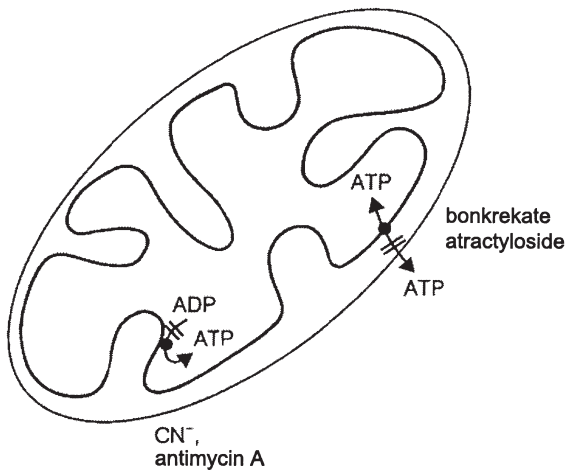
**Figure 95** Dinitrophenol (DNP) as a protonophore. DNPH (From Ref. 4.)



**Figure 96** Translocase inhibitors.

Bongkreikic acid, an antibiotic from *Pseudomonas cocovenans*, is produced in infected coconut products (Indonesian, *bongkrek*). Bongkreikic acid inhibits the translocation in a noncompetitive manner. Mitochondria are depleted of ATP if oxidative phosphorylation is blocked by inhibitors of the respiratory chain (e.g.,  $\text{CN}^-$  or antimycin) and ATP translocase is simultaneously knocked off. It is not sufficient to inhibit only the respiratory chain, because the translocase would redirect cytosolic ATP (substrate-chain phosphorylation) into the mitochondrial matrix (Fig. 97). By this approach Schatz and coworkers (192) showed for ATP-deficient yeast mitochondria that cytoplasmic precursor proteins are still synthesized for the mitochondria. However, the organelle import and the concomitant processing to the final size are prevented. Mitochondrial import is blocked by the mitochondrial ATPase inhibitor oligomycin and by valinomycin/ $\text{K}^+$ , which leads to the breakdown of the membrane potential (193). However, if mitochondria are energized by a respiratory substrate (e.g., succinate) in the presence of oligomycin, then the import is not inhibited by antibiotics. This is clear





**Figure 97** Adenosine triphosphate (ATP) depletion of mitochondria by specific inhibition of the two main routes for ATP supply: KCN and antimycin A block oxidative phosphorylation. Bongkrekeic acid and atractyloside prevent the supply of ATP (from glycolysis) from the cytoplasm by ATP/ADP-translocase. ADP, adenosine diphosphate. (From Ref. 192.)

evidence for the crucial role of the electrochemical gradient in vectorial transport.

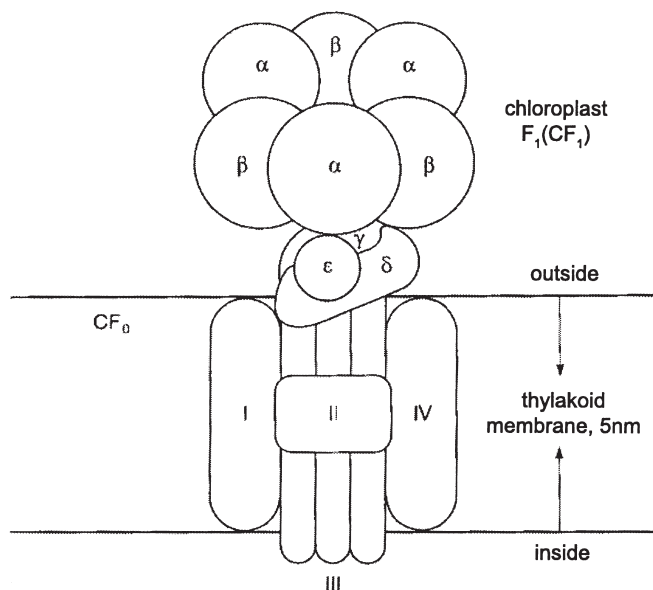
## XXII. CHLOROPLASTS

### A. Inhibition of Photosynthesis

There are numerous inhibitors of photosynthesis. Most are herbicides, e.g., electron transport inhibitors, pigment synthesis inhibitors, as well as a number of bacterial and fungal phytotoxins. These inhibitors are dealt with in separate chapters of this book (cf. Chapter 7, Herbicides and Chapter 10, Host Parasite). A smaller number of antibiotics are available for the inhibition of photosynthesis than for inhibition of respiration. This may be due to the fact that the search for antibiotics has focused so far on compounds that impair nonphotosynthetic organisms (e.g., fungi and most bacteria).

### B. Inhibition of the Chloroplast F-Adenosine Triphosphatase

The mechanism of photophosphorylation resembles in principle oxidative phosphorylation: ATP synthesis becomes energized by an



**Figure 98** F-adenosine triphosphatase (F-ATPase) model of the chloroplast. (From Ref. 81.)

electrochemical gradient of protons across the thylakoid membrane. Essential differences from mitochondrial ATP synthesis are (a) that the photosynthetic electron transport is driven by light and (b) that the protons are pumped from the outside into the thylakoid lumen. Synthesis of ATP is coupled to the efflux of the protons by a membrane-bound ATP synthase.

Chloroplasts are equipped similarly to mitochondria, with an F-ATPase (=  $F_0F_1$ -ATPase) with an integral  $F_0$  moiety, which extends into the stroma (Fig. 98). Coordinated regulation warrants that its activity is switched off in the dark when the proton gradient is more shallow, in order to prevent an inverse reaction (hydrolysis of ATP).

In contrast to activity of the mitochondrial ATP synthase, the activity of the chloroplast enzyme is latent and requires activation. It involves a protease that produces  $Ca^{2+}$ -dependent ATP synthase activity or light in presence of sulfhydryl reagents triggering  $Mg^{2+}$ -dependent activity. It was suspected for a long time that the chloroplast ATP synthase is insensitive to oligomycin. Bouthyette and Jagendorf (194) reported that oligomycin inhibition is observed after activation, which also

depends in a complex way on other factors (season, relative atmospheric humidity, etc.).

As in the case of the mitochondrial F-ATPase, azide inhibits the  $F_1$  unit of the chloroplast enzyme. The fungal antibiotic venturicidin, which blocks the mitochondrial ATPase, also inhibits specifically the proton flux through  $CF_o$  (195) of the chloroplast ATPase, comparably to DCCD, triphenyltin, and tributyltin. Phlorizin and Dio 9, however, block  $DF_1$ .

Tentoxin is a cyclic tetrapeptide [ $p(\text{cyclo(L-leucyl-N-methyl-trans-dehydrophenylalanyl-glycyl-methyl-L-alanyl)})$ ] from the fungus *Alternaria tenuis*. Tentoxin induces chlorosis by disturbing plastid development (196). Although the toxin inhibits the chloroplast ATPase noncompetitively by binding to the  $CF_1$  factor, the main effect may be photo bleaching (cf. later discussion) due to diminished chlorophyll accumulation.

### C. Decouplers

Since the ATP synthesis is based on a chemiosmotic mechanism in chloroplasts and mitochondria, all substances that decrease the electrochemical potential across the thylakoid membrane inhibit ATP synthesis. In most cases the decoupling effect is similar. Distinct differences are due to the fact that the pH gradient  $\Delta \text{pH}$  in chloroplasts mainly contributes to the electrochemical potential difference. Permeable ions and electrogenic ionophores have, in contrast to mitochondria, little influence on photophosphorylation.

Substances that destroy the pH gradient are strong decouplers in chloroplasts. Nigericin (cf. previous discussion) catalyzes an electrically neutral exchange of  $K^+$  for  $H^+$  and therefore diminishes the  $\Delta \text{pH}$ .

On the other hand, decoupling by high concentrations of  $NH_4^+$  is based on membrane damage as a result of excessive swelling. Disturbances of the thylakoid structure are assumed to be the reason for the inhibition of the trans-thylakoidal  $\Delta \mu H$  by the local anaesthetic dibucaine (197).  $NH_3$  interferes with the  $O_2$  production by binding to the  $O_2$ -forming complex on the oxidizing side of photosystem II (PS II).

$Ca^{2+}$  and a calmodulinlike protein regulate the electron transport in chloroplasts. In addition  $Ca^{2+}$  regulates photophosphorylation (198); thereby calmoduline antagonists such as phenothiazine and pimozide or  $Ca^{2+}$  chelators block the electron transport and the formation of proton gradients in the context of noncyclic and cyclic phosphorylation. On the other hand,  $Ca^{2+}$  antagonists such as  $LaCl_3$  just interfere with the electron transport.

### XXIII. INHIBITION OF THE CELL WALL SYNTHESIS

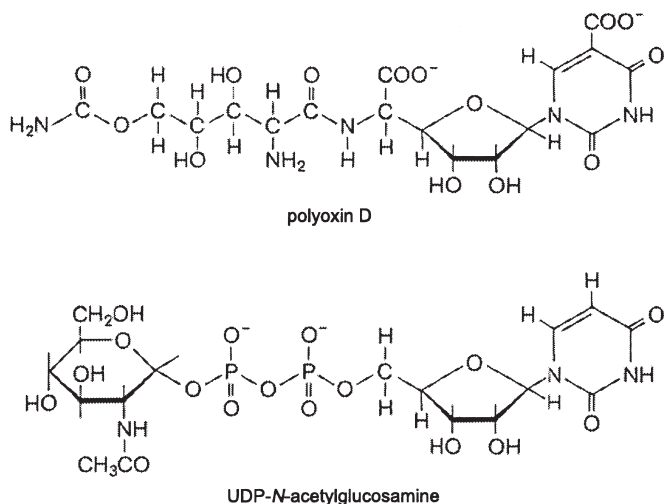
The cell wall provides protection and mechanical strength to the plant cell. It prevents bursting of the protoplast in hypotonic solutions and still allows growth in early stages of development. Two main components are involved in the assembly of plant cells: fibrillar elements, whose arrangement determines the cell form, and an amorphous matrix (cf. Chapter 1, Plant Morphology). This construction principle can be compared with that of reinforced concrete or glass fiber-reinforced synthetic resin plates.

Interference with cell wall synthesis inevitably leads to the loss of cell vitality. Particularly fungi can be selectively damaged, because of their unusual cell wall. Chitin,  $\beta$ -glucane, and cellulose are predominantly involved in the structure of the fibrillar elements. The matrix consists of amorphous polysaccharides and protein-polysaccharide complexes. Considerable quantitative and qualitative differences exist for different fungal groups. Chitin, for instance, appears only in the septum of yeasts, which separates mother from daughter cells. Mannan plays a predominant role. Chitin is replaced by cellulose in oomycetes. Polyoxin D, a nucleoside antibiotic from *Streptomyces cacaoi*, competitively inhibits the chitin synthetase. The structure shows some similarity to that of the substrate UDP-*N*-acetylglucosamine (Fig. 99).

The rather narrow spectrum of effects probably depends on difficulties in penetration. Polyoxin D damages those regions where active chitin synthesis takes place, e.g., in hyphal tips or sites of bud formation in yeasts. The strength of the cell wall is lost. Hyphal tips swell and burst. In yeasts plasma leaks from the contact site between mother and daughter cells. Polyoxins were used for the treatment of the black spot disease of pears in Japan. Human pathogenic fungi are hardly affected.

Griseofulvin (from *Penicillium griseofulvum*) probably interferes with chitin synthesis, too. It disturbs the assembly of cell walls containing chitin and causes characteristic curling and wall thickening at hyphes (cf. Fig. 100). Tunicamycin blocks mannan synthesis by inhibiting *N*-glycosylation (cf. above) and therefore interferes with the assembly of mannan-containing cell walls, which are characteristic of yeasts.

A secondary metabolite that is found in fresh plant tissues in its bound form (trans-*O*-glucosyl cinnamon acid) is coumarin. After cell injury it is enzymatically converted to the lactone compound. Coumarin (Fig. 101) is a volatile compound that is released through the leaf surface. Coumarin determines, for instance, the smell of fresh hay. The compound blocks cellulose synthesis of several plant species. The assembly of the primary and secondary cell walls is probably controlled by a common coumarin-sensitive



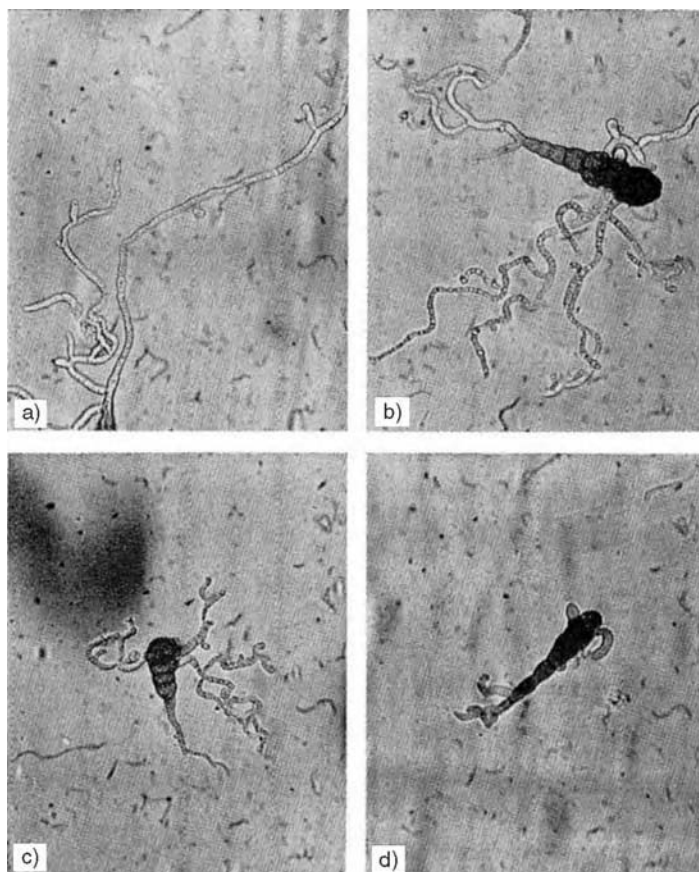
**Figure 99** Polyoxine and uridine diphosphate- (UDP)-*N*-acetylglucosamine.

step. Coumarin and its derivatives interfere with several other metabolic and developmental processes (200). Therefore, applications as a specific inhibitor of the cellulose synthesis are limited.

## XXIV. SIGNAL TRANSDUCTION CHAIN

Signal transduction chains (STCs) play a key role in inter- and intracellular communication and are therefore crucial for developmental processes. A wealth of information is known from medical research. Several signaling pathways have been examined in great detail. In principle four classes of STCs can be discriminated, starting with the following cell surface receptors: (a) G protein-coupled receptors, (b) ion channel receptors, (c) tyrosine kinase-linked receptors, (d) receptors with intrinsic enzymatic activity. In addition, STCs originating in intracellular receptors, such as steroid receptors, are known.

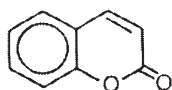
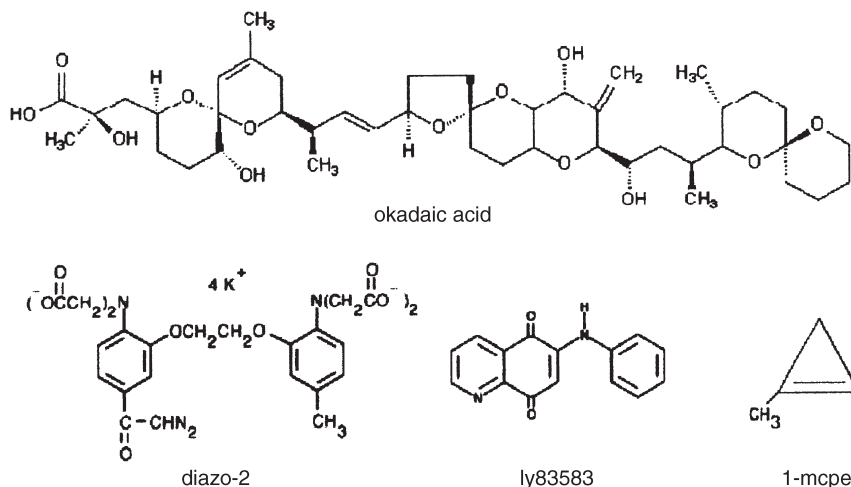
In plants, knowledge of STCs is much less complete although research on *Arabidopsis* sp. has significantly contributed to a better understanding. As the main tools in STC research and inhibition mutants are valuable for functional investigations. Examples of the inhibition of hormone STCs are given in the following.



**Figure 100** Effect of griseofulvin on spore germination of *Alternaria solani*: a, Control lacking griseofulvin; b, 0.6 µg/ml griseofulvin, bent hyphae; c, 1.25 µg/ml griseofulvin, growth inhibition, bent hyphae, and redundant branches; d, 2.5 µg/ml griseofulvin, fundamental inhibition of hyphae growth. (From Ref. 199.)

#### A. Receptor Inhibition at the Perception Site of the Ethylene Signal Transduction Chains

In *Arabidopsis* sp., molecular recognition of ethylene is mediated by a family of five receptors encoded by the genes *ETR1*, *ERS1*, *ERS2*, *ETR2*, and *EIN4*. *ETR1* reversibly binds ethylene and has a striking similarity to receptor histidine kinases of the two-component signaling system that is known for prokaryotic organisms (201).

**Figure 101** Coumarin.**Figure 102** Inhibitors of signal transduction chains.

The ethylene antagonist 1-methylcyclopropene (1-MCP; Fig. 102) competitively inhibits ethylene binding in genetically engineered yeast expressing *ETR1* and *ERS1*, which is also an ethylene-binding protein (202). In addition, 1-MCP inhibits ethylene-induced changes in the growth of *Arabidopsis* sp. 1-MCP has been shown to inhibit ethylene-induced ripening and senescence at very low concentrations. Its effectiveness as a competitive inhibitor is at least one order of magnitude better than that of other unsaturated cyclic olefins, such as trans-cyclooctene and 2,5-norbornadiene (203). The effect of 1-MCP on the triple response of *Arabidopsis* sp. seedlings was investigated by Hall and associates (204). The ethylene-mediated triple response is characterized by an inhibition of hypocotyl and root elongation, radial swelling, and formation of an exaggerated apical hook. The inhibitor effect reversed the triple response; 1-MCP does not interfere with the triple response effect in *Arabidopsis* sp. seedlings that carry the *ctr1-2* mutation, which leads to a constitutive ethylene response in the presence or absence of ethylene. Consequently, the effects of 1-MCP occur upstream of *ctr1-2* in the ethylene signal transduction pathway with a direct effect on the receptor.

## B. Inhibitors of the Gibberellin- and Absciscic Acid-Triggered Signal Transduction Chains

Between germination and establishment of phototrophic growth, seedlings rely on nutrients stored in the seed. In Gramineae, aleurone cells help to mobilize storage material. This mobilization by aleurone cells is strongly correlated with the phytohormone gibberellin (GA) produced de novo by the embryo, and with a decline of abscisic acid (ABA) level in the endosperm (205). The relative ease of isolating aleurone tissue from other cell types, combined with the specificity of the responses of these cells to GA and ABA, has made aleurone a model system for studying the molecular effects of these hormones. A comprehensive review of the STCs of GA and ABA was published in 2000 by Lovegrove and Hooley (206).

The plant hormones GA and ABA regulate gene expression, secretion, and cell death in aleurone. It is assumed that GA perception takes place at the plasma membrane, whereas ABA acts at both the plasma membrane and in the cytoplasm, although GA and ABA receptors have yet to be identified. A range of downstream-signaling components and events have been implicated in GA and ABA signaling in aleurone. These include the  $G_\alpha$  subunit of a heterotrimeric G protein, a transient increase in cyclic GMP (cGMP) levels,  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent events in the cytoplasm, reversible protein phosphorylation, and several promoter cis-elements and transcription factors (206).

### 1. Inhibitors of Cytoplasmic $\text{Ca}^{2+}$

The earliest event after GA treatment of aleurone is an increase in cytoplasmic calcium concentration ( $[\text{Ca}^{2+}]_i$ ) within 4–6 h after GA application (207). If GA is present continuously, it causes sustained elevations in  $[\text{Ca}^{2+}]_i$  concentration that lead to increases of 100–500 nM above a resting level of 100–250 nM. It is suggested that GA alters  $\text{Ca}^{2+}$  flux at the plasma membrane (208, 209). These alterations in  $[\text{Ca}^{2+}]_i$  concentration indicate a potential signaling role. The fact that calmodulin (CaM) protein levels are stimulated two- to fourfold by GA and reduced by ABA (210) suggests that a  $\text{Ca}^{2+}$ –CaM system may operate.

To identify  $\text{Ca}^{2+}$ - and CaM-dependent and -independent signaling events in aleurone, the  $[\text{Ca}^{2+}]_i$  and CaM levels can be modified by microinjecting  $\text{CaCl}_2$ , caged  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  chelators, CaM, and CaM antagonists into aleurone protoplasts (209). For instance, the GA-induced increase in  $[\text{Ca}^{2+}]_i$  can be blocked by using the  $\text{Ca}^{2+}$  chelator diazo-2 (Fig. 102). Diazo-2 inhibits GA-induced  $\alpha$ -amylase secretion but does not significantly affect *Amy:GUS* expression. CaM antagonists also inhibit



$\alpha$ -amylase secretion. A study with rice aleurone (211) concluded that  $\text{Ca}^{2+}$  and CaM are important intermediates in the GA induction of gene expression.

## 2. Inhibitors of Cyclic Guanosine Monophosphate

It is likely that a transient rise in cGMP is an important component in the signaling necessary for GA-regulated gene expression,  $\alpha$ -amylase secretion, and programmed cell death (212, 213). Radioimmunoassay has been used to measure the cGMP in barley aleurone layers (212). Controls and layers treated with 5 mM ABA contained the same amounts of cGMP over a 6-h period. Gibberellin-treated layers showed an approximately threefold rise in cGMP 2 h after GA addition, which returned to control levels within a further 2 h. On the basis of measurements of  $[\text{Ca}^{2+}]_i$  in wheat aleurone layers, the alterations in cGMP probably occur after the rise in  $[\text{Ca}^{2+}]_i$ . Nevertheless, they certainly precede the increase in transcription of  $\alpha$ -amylase genes. An inhibitor of guanylyl cyclase, LY83583 (Fig. 102), reduces the increase in cGMP and inhibits the induction of  $\alpha$ -amylase and mRNAs of the transcription factor GAMYB, as well as  $\alpha$ -amylase protein secretion. LY83583 also prevents DNA degradation and cell death (213).

## 3. Inhibitors of Protein Kinase Cascades

Reversible protein phosphorylation is a universal signal-transducing mechanism. There is molecular and pharmacological evidence for the involvement of protein kinase cascades in GA and ABA action. The ABA treatment of wheat and barley aleurone induces the expression of a serine/threonine protein kinase, PKABA1 (214). A possible role for this kinase in ABA inhibition of GA-regulated gene expression is suggested from experiments in which constitutive overexpression of PKABA1 strongly inhibits GA induction of  $\alpha$ -Amy1,  $\alpha$ -Amy2, and cysteine proteinase promoter:GUS constructs.

Protein kinase substrate peptides have been microinjected into barley aleurone protoplasts in order to compete with the endogenous protein phosphorylation targets and block kinase cascades (215). The 15mer peptide syntide-2 selectively inhibits GA induction of  $\alpha$ -Amy2:GUS,  $\alpha$ -amylase secretion and protoplast vacuolation. The peptide does not prevent GA stimulation of  $[\text{Ca}^{2+}]_i$ , indicating that it is acting downstream of this early event in GA signaling. Remarkably, syntide-2 is a specific substrate for mammalian CaM-kinase II. To date, there is no direct evidence for a CaM-kinase II in plants. Therefore, the identity of the

aleurone kinase or kinases blocked by syntide-2 is not clear. Nevertheless, on the basis of a series of in vitro phosphorylation and protein-labeling assays, a putative  $\text{Ca}^{2+}$ -activated CaM-like domain protein kinase (CDPK) that interacts with syntide-2 has been identified in barley aleurone cytosol (215). At this stage it is not known whether syntide-2 blocks one or more events in the GA-signaling and response pathway. Syntide-2 may interfere with CDPK regulation of the SV-type cation channel; it is not known what effect this would have on signaling. Syntide-2 does not affect ABA action, suggesting that the kinase acting on it does not cross-talk with ABA signaling.

Pharmacological studies have provided further evidence for kinases and phosphatases in GA and ABA signaling. Okadaic acid (OA; Fig. 102), a polyether derivative of a C-38 fatty acid from the microorganism *Prorocentrum lima*, inhibits GA-induced increases in  $[\text{Ca}^{2+}]_i$ ,  $\alpha$ -amylase mRNA,  $\alpha$ -amylase secretion, and aleurone cell death at concentrations that inhibit the activity of serine/threonine protein phosphatases (PPs) PP1 and PP2B (216). The effect of OA on the increase of  $[\text{Ca}^{2+}]_i$  caused by GA suggests that a reversible phosphorylation event is an essential part of this particular  $\text{Ca}^{2+}$  signature, but not of the  $[\text{Ca}^{2+}]_i$  increase caused by hypoxia (216). Although details are currently not known, it may prove useful to search for protein phosphorylation events that occur within minutes after GA treatment. Because OA also inhibits GA induction of  $\alpha$ -amylase gene expression and cell death—events independent of the elevation of  $[\text{Ca}^{2+}]_i$ —OA could be acting in several ways. In barley aleurone protoplasts, OA partly inhibits ABA-induced *PHVA1* gene expression (216). Tyrosine and serine/threonine phosphatase inhibitors prevent ABA-induced *RAB* gene expression, leading to tyrosine hyperphosphorylation of two 40-kd polypeptides (217). A current hypothesis is that ABA regulation of *rab16* gene expression in barley aleurone involves rapid stimulation of a putative mitogen-activated protein kinase (MAP kinase) via a tyrosine phosphatase (218).

In summary, a range of molecular and pharmacological data support the hypothesis that protein kinases and phosphatases are involved in GA and ABA signaling in aleurone. The enzymes involved, their targets, and their precise relationships to GA-stimulated events are poorly defined. For ABA signaling, the apparent role of reversible protein phosphorylation in aleurone is supported by molecular genetic evidence that two OA-insensitive serine/threonine phosphatases are involved in ABA signaling in *Arabidopsis* sp. (219). In the case of GA, investigations have highlighted that the role of  $[\text{Ca}^{2+}]_i$  in GA regulation of gene expression is not fully understood.

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# 9

## Metabolism and Elimination of Toxicants

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### I. INTRODUCTION

Plant toxicants may include endogenous substances such as secondary plant metabolites (e.g., phenolics, alkaloids, flavonoids) or xenobiotic compounds. Xenobiotics (from Greek words meaning “foreign chemicals”) are natural or synthetic substances that cannot be utilized by plants for energy-yielding processes. Chemicals classified commonly as xenobiotics include pesticides and air pollutants. Higher plants are exposed to xenobiotics either deliberately, i.e., pesticide application, or accidentally, as a result of industrial emissions or misuses of agrochemicals.

The term *metabolism* is used to describe transformation of agrochemicals in or on plants by biological or abiotic processes. Higher plants can transform endogenous and xenobiotic toxicants through activation or detoxification reactions (1, 2). The specific functional groups or linkages (i.e., hydroxyl, alkyl, amino, nitro, amide, carboxyl, nitrile, and halogens) found in the molecules of endogenous or xenobiotic toxicants are susceptible to chemical, physical, and enzymatic transformations. The rate of toxicant biotransformations is dependent upon the plant species; plant selectivity of agrochemicals (e.g., herbicides) and differential response to air pollutants are the result. Enzymatic transformation is by far the major means of detoxification, and toxicants may be altered through oxidative, reductive, or hydrolytic reactions or be activated to serve as reactive intermediates for the subsequent conjugation of the parent



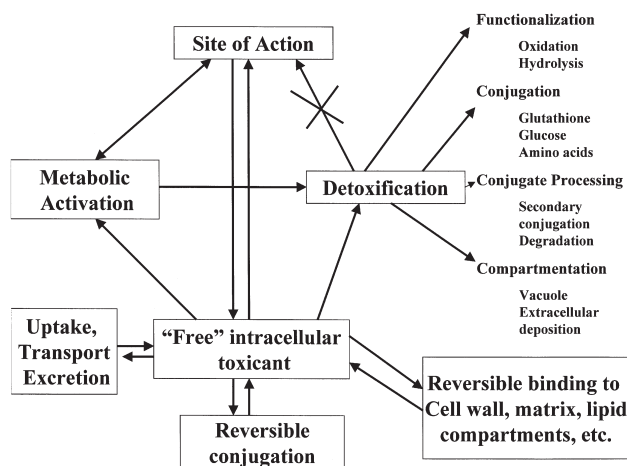
molecules with endogenous substances such as sugars, glutathione, or amino acids (1–4).

During the past four decades, most investigations of the metabolism of agrochemical toxicants in plants have been driven mainly by the need (a) to fulfill registration and regulatory requirements and (b) to understand the mechanism of plant selectivity of old and new agrochemicals, mostly herbicides. As a result, an enormous body of scientific literature is available on the metabolism of herbicides and other xenobiotics in plants. For detailed information on the metabolism of agrochemicals and the methodology available for studying metabolism of toxicants in higher plants, the reader is referred to past or recent reviews and books available on this subject (2–16). Because of space limitations, this chapter provides a brief overview of the principles underlying the stepwise processes involved in the metabolism and elimination of agrochemical toxicants (mainly herbicides) in plants.

## II. INTEGRATED PATHWAYS OF TOXICANT METABOLISM IN PLANTS

Plants metabolize toxicants through a multiphase process that converts the parent molecule to more polar products and insoluble bound residues (15, 16). By analogy with the metabolism of xenobiotics in mammalian and insect systems, the metabolism of toxicants in plants has been viewed as a stepwise process of three major phases (15, 16). Primary or phase I metabolism converts biologically active toxicants into less active chemicals via hydrolysis and oxidation, but occasionally into more toxic metabolites through bioactivation. Secondary or phase II metabolism includes conjugation processes that link the products of phase I with plant constituents such as sugars, amino acids, glutathione, and other small molecules. Tertiary or phase III metabolism converts phase II products into secondary conjugates or insoluble bound residues.

More recently, a four-phase process has been proposed for the integration of the pathways of herbicide metabolism in plants (3, 16). The first two phases, termed *functionalization* and *conjugation*, respectively, are similar to the primary and secondary phases described in the previous paragraph. Phase III includes the processing of conjugates formed in phase II by means of secondary conjugation (i.e., malonic acid conjugation) or degradation of glutathione conjugates by peptidases to cysteine conjugates. The fourth phase, described as *compartmentation*, includes the processes of pump-mediated transport of toxicant conjugates to the vacuole of plant cells and/or the extracellular deposition of toxicant metabolites (i.e., cell wall).



**Figure 1** Schematic overview of toxicant metabolism in plants. (Modified from Ref. 1.)

In contrast to mammalian systems, plants lack well-defined excretion systems for the complete elimination of toxicants and their metabolites. Nevertheless, root exudation or volatilization from leaf margins (i.e., guttation) has been shown to contribute partially to the elimination of some acidic herbicides (5).

The integrated pathways of herbicide metabolism in plants are summarized in Fig. 1. It should be emphasized that the four phases of the integrated pathways may not be involved in the metabolism of all toxicants or herbicides in plants. The reversible binding of a toxicant to lipid bodies, cell walls, or other compartments decreases the pool of “free” intracellular toxicant exposed to the transformations of each phase. Furthermore, the parent toxicant may be a protoxicant that must be first converted to the active molecule through bioactivation in treated plants. Proherbicides may penetrate into the plant more readily (e.g., ester formulations of acidic herbicides) and once inside the cell may be converted to the herbicidally active acidic structure (1).

The enzymatic systems involved in herbicide detoxification by plants and the types of reactions carried out are quite similar to the enzymes and reactions found in the biosynthetic pathways of secondary plant metabolites. For example, common metabolic reactions such as aryl or alkyl hydroxylations mediated by cytochrome P450-dependent mono-oxygenases (P450s) and subsequent conjugations of the hydroxylated products with glucose by uridine diphosphate glucose– (UDPG)-glucosyltransferases are involved in the metabolic pathways of both herbicides and endogenous

substances such as anthocyanins (17, 18). These parallels suggest that herbicide detoxification in plants may be carried out by enzymes with normal endogenous roles in plant secondary metabolism.

Several inducers, such as mechanical wounding, infection, and stress conditions, including chemical treatment, may trigger all enzymes involved in the synthesis of “defense” compounds such as many secondary plant metabolites (19). The inducibility of the enzymatic systems detoxifying xenobiotics in plants and animals by the same chemicals (e.g., phenobarbital, ethanol, etc.) indicates that regulation mechanisms may have been conserved along with the catalytic function of these enzymes during evolution (19). Environmental factors such as temperature, light intensity, nutrition, and water stress play an important role in the metabolism of specific toxicants in plants (5, 7). In addition, plant species differences coupled with the degree of plant maturity or age influence the levels and/or activities of plant enzymes involved in the detoxification of toxicants (5, 7).

### **III. PRIMARY METABOLISM OF TOXICANTS IN PLANTS**

The primary metabolism of toxicants in plants often results in the formation of metabolites with reduced or modified phytotoxicity, increased polarity, and predisposition of the parent molecule to serve as a substrate for conjugation reactions of phase II. The presence of necessary substituents in the molecules of some toxicants (e.g., chlorine, amino group, or amide bonds) allows them to conjugate directly with cellular substances. Most herbicides, however, must be first converted into metabolites that can conjugate with sugars, glutathione, or amino acids. Most of the reactions of the primary metabolism of toxicants in plants are oxidative, catalyzed by cytochrome P450 mono-oxygenases, or hydrolytic, catalyzed by esterases. Some reductive reactions are known to occur, but they are not considered very important in the primary metabolism of toxicants in plants.

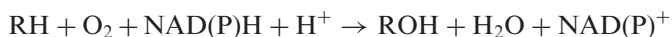
#### **A. Toxicant Metabolism Mediated by Plant Cytochrome P450s**

##### **1. Properties of Plant P450s**

The microsomal cytochrome P450-dependent mono-oxygenases (P450s; EC 1.14.14.1) are the most versatile of the enzymes utilized in the metabolism and detoxification of herbicides and other xenobiotics in plants (19–24).

They usually exist as multigene families of membrane-bound enzymes with varying and overlapping substrate specificity capable of catalyzing the oxidation of a diverse range of compounds (21–24). They are important in the metabolism and detoxification of xenobiotics as well as the metabolism and regulation of endogenous substrates (25–30).

In most cases, P450s function as mono-oxygenases, by binding and activating molecular oxygen, incorporating one of its atoms into an organic substrate (RH), and reducing the second atom to water, as shown in the following reaction:



The P450-mediated catalysis usually results in substrate hydroxylation, but epoxidation, heteroatom dealkylation, deamination, isomerization, C-C or C=N cleavage, dimerization, ring formation, dehydration, dehalogenation, or reduction have been also reported with plant or microbial P450s (30).

The P450s usually consist of two essential elements, a cytochrome P450 substrate binding component, which acts as the terminal oxidase, and the flavin adenine dinucleotide (FAD)/flavin mononucleotide (FMN) dependent reduced nicotinamide adenine dinucleotide phosphate–(NADPH)–cytochrome P450 reductase component that transfers electrons from NADPH to the cytochrome P450 (21–23). In plants as well as other eukaryotic organisms, P450s and reductases are usually microsomal membrane-bound proteins, exposed to the cytosol. Soluble forms of P450s, coupling P450 and reductase in a single fusion protein, have also been found in bacteria and fungi (31). A second type of P450, which requires ferredoxin in addition to flavoprotein for the transfer of electrons from NAD(P)H to the terminal P-450, has been identified in prokaryotes and animal mitochondria. A third class of P450s does not require an auxiliary redox partner or molecular oxygen to catalyze the rearrangement of hydroperoxides. Plant P450s of this type are located in the plastids (30).

Reduced P450s can bind carbon monoxide instead of oxygen, forming a complex that shifts the Soret absorption maximum between 447 and 452 nm (30). As a result, the enzymatic reaction is blocked, but inhibition can be partly reversed by light, with a maximum of efficiency around 450 nm. A second species absorbing at 420 nm, which is considered to be an inactive form of P450, is often simultaneously detected. When a substrate binds the active site, the result is a so-called type I spectrum with a peak near 390 nm and a trough around 420 nm (30). The addition of

P450-inhibiting compounds produces a type II absorption spectrum, which has a peak at 430 nm and through at 390–400 nm (30). These absorption changes indicate shifts in the spin equilibrium and redox potential of the cytochrome.

## 2. Functions of Plant P450s and Their Genes

The P450s are encoded by a superfamily of genes. More than 500 P450 genes have been sequenced from many organisms and are available (32). The P450s genes are grouped into more than 150 families according to the amino acid sequences of the deduced proteins. With a few exceptions, based on phylogenetic considerations, protein sequences within a given family are 40% identical. When two sequences are more than 55% identical, proteins are designated as members of the same subfamily. The nomenclature of P450 genes uses the prefix *CYP*, numbers designating families, and letters designating subfamilies. Plant P450s correspond to the families *CYP71* to *CYP99*, then *CYP701* and above (21, 32).

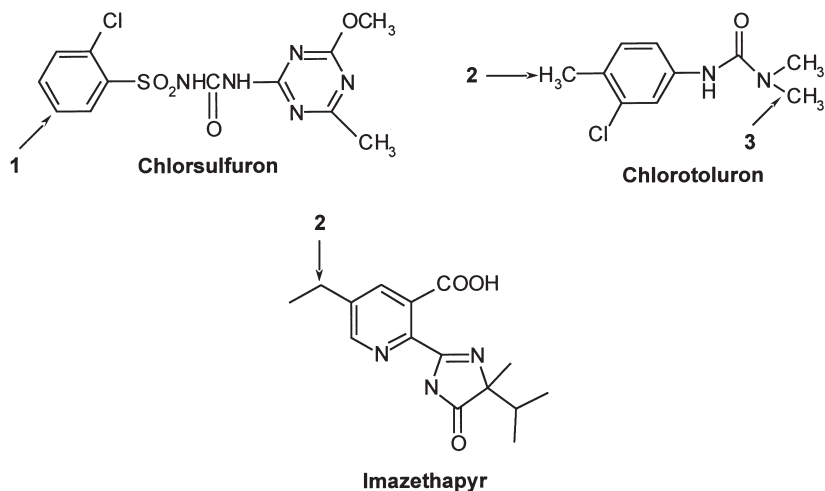
After the cloning of the first plant P450 gene, *CYP71A1*, in the early 1990s, more than 200 genes have been registered in plants (30). The apoprotein sequences (45 to 60 kd) are highly variable, but their three-dimensional structures seem to be somewhat similar. The core surrounding the heme prosthetic group, the oxygen binding site, and the oxygen activation site are highly conserved parts of all P450 proteins. Most P450s seem to be anchored to membranes primarily via a 30- to 50-amino-acid *N*-terminal segment (30).

At present, the function of most of the plant P450 genes sequenced remains unknown (30). Natural substrates of plant P450s include plant secondary metabolites such as phenylpropanoids, isoprenoids, alkaloids, plant growth regulators, amino acid derivatives, sterols, and fatty acids. Selected plant P450s are capable of metabolizing xenobiotic toxicants, mainly herbicides.

## 3. Role of P450s in Herbicide Metabolism

Plant P450s have been implicated in the metabolism of numerous herbicides (25–30). The P450-mediated reactions involved in herbicide metabolism include *N*-dealkylation of phenylureas; aryl hydroxylation of phenylureas, sulfonylureas, imidazolinones, phenoxyalkanoates, flumetsulam, bentazon, and diclofop; and ring-methyl hydroxylation of flumetsulam, metolachlor, and chlortoluron. The chemical structures of selected herbicides oxidized by plant cytochrome P450s are shown in Fig. 2.

Studies conducted by Zimmerlin and Durst (33–35) with wheat microsomal P450s provided evidence for the existence of an enzyme capable



**Figure 2** Chemical structures of herbicides metabolized by P450-mediated reactions in plants: 1, Ring hydroxylation; 2, alkyl hydroxylation; 3, dealkylation.

of mediating both the hydroxylation of fatty acids and the aryl hydroxylation of the herbicide diclofop-methyl. The observed competitive inhibition between laurate hydroxylation and diclofop aryl hydroxylation indicated that a common enzyme was responsible for both P450 activities (33–35).

Because of the low levels of P450s in plants and the inherent difficulties in their purification, our knowledge of the biochemical and molecular biological characteristics of plant P450s is limited (21–23). The complementary deoxyribonucleic acid (cDNA) sequences encoding plant P450s have been published, but not much is currently known about genes encoding plant P450s involved in herbicide detoxification and their regulation by herbicide modulators (22, 24, 26, 30). Constructs derived from the fusion of the rat *CYP1A1* cDNA and the yeast NADPH P450 reductase cDNA conferred chlorotoluron resistance in transgenic tobacco and potato plants due to enhanced herbicide metabolism (36). In another study, transgenic tobacco engineered with a chloroplast-targeted bacterial *CYP105A1* gene metabolized and activated the sulfonylurea proherbicide R7042 (37).

#### 4. Regulation of Plant P450s

The regulation of the plant P450 gene/enzyme system is poorly understood. The expression of P450 enzymes and their genes follows a developmental

regulation pattern, which is tissue-specific. Physicochemical (light, osmotic stress, wounding) and physiological (infection, aging, hormones) factors and treatment with xenobiotics (herbicides, safeners, ethanol, phenobarbital, or aminopyrine) have been shown to induce plant P450s (19).

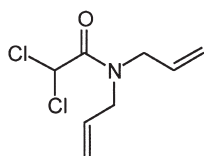
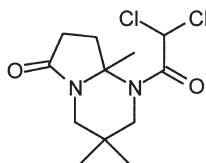
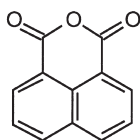
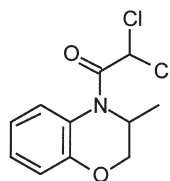
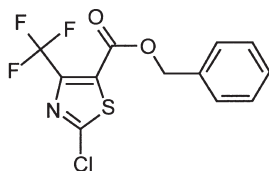
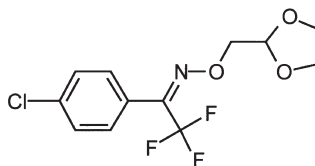
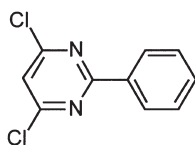
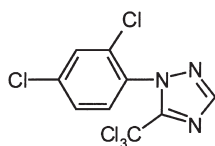
Plant P450 enzymes can be inhibited by mechanism-based inactivators such as 1-aminobenzotriazole (ABT), heterocyclic molecules (e.g., imidazole, pyrimidine, triazole derivatives), or methylenedioxy compounds (e.g., piperonyl butoxide). Some of these compounds inhibit a broad range of enzymes, whereas others seem to be more selective.

*Safeners and Plant 450s.* Safeners (also known as *antidotes*) are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism (38–45). At present, commercialized safeners are chemical compounds that structurally resemble the herbicides that they antagonize on selected crops (38–45).

Grass crops protected by herbicide safeners are moderately tolerant to the antagonized herbicides, and all safeners are most effective when applied before or simultaneously with their respective herbicides. In practice, safeners are applied either to the crop seed before planting (*seed safeners*) or to the soil or crop together with the herbicides in a single formulation package (38–45). Seed safeners include mainly the safeners used with grain sorghum and the safener naphthalic anhydride (NA), which can be used with maize or other crops. With the exception of NA, all maize safeners contain a dichloromethyl group, are effective against thiocarbamates and chloroacetanilides, and are applied as prepackaged formulated mixtures with the respective herbicide (40).

Safeners are known to induce the activity of several plant P450s involved in the metabolic detoxification of chloroacetanilide, aryloxyphenoxypropionate, sulfonylurea, imidazolinone, and sulfonamide herbicides in protected grass crops (26–29). In fact, NA and other safeners are used commonly to induce cytochrome P450s in microsomes extracted from grass species such as maize and wheat that are known to metabolize specific herbicides *in vivo* rapidly (26–29). Figure 3 presents the chemical structures of selected herbicide safeners discussed in this chapter.

At present, not much is known about genes encoding plant P450s involved in herbicide detoxification and their regulation by herbicide safeners (24). There is some indirect evidence that the induction of P450 isoforms by safeners may be exerted through enhanced gene expression, a process much like the safener-mediated induction of plant glutathione *S*-transferases (19, 24, 26, 29). However, the exact mechanism by which safeners regulate the activity of plant P450s metabolizing herbicides is currently unknown (19, 26, 29).

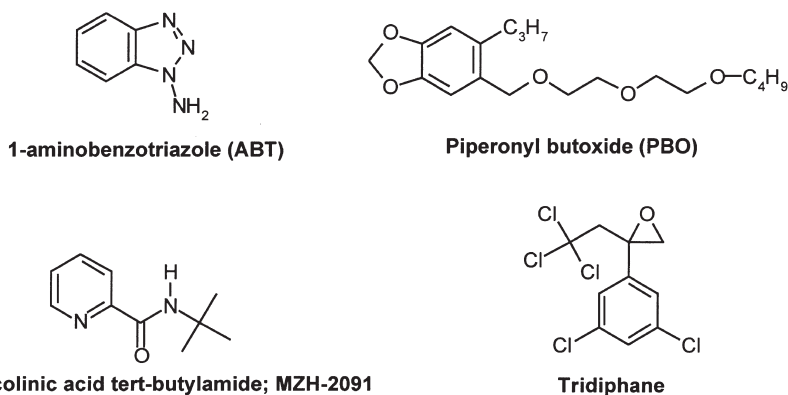
**Dichlormid****BAS-145138****Naphthalic anhydride (NA)****Benoxacor****Flurazole****Fluxofenim****Fenclorim****Fenchlorazole-ethyl**

**Figure 3** Chemical structures of selected herbicide safeners discussed in this chapter.

*Synergists and Plant P450s.* Piperonyl butoxide (PBO) and sesamex, two inhibitors of P450s, have been used as effective insecticide synergists for several years (46, 47). Similarly, many known P450 inhibitors inhibit oxidative herbicide metabolism and synergize the activity of some herbicides (1, 45–52).

Both ABT and PBO (Fig. 4) are suicide substrates that bind irreversibly to cytochrome P450 and therefore inhibit its enzymatic activity (1, 53–55). The mechanism of PBO binding to P450 has been described as an  $\pi$ - $\sigma$  bonding, after the enzymatically catalyzed formation of the carbene structure (54). The stable PBO–P450 complex formed can no longer bind





**Figure 4** Chemical structures of selected herbicide synergists discussed in this chapter.

carbon monoxide. 1-Aminobenzotriazole (ABT) has been shown to synergize many herbicides that are metabolized by *N*-dealkylation and aryl hydroxylation reactions (53). The generally accepted conclusion is that synergism or resistance reversal by an ABT-herbicide combination indicates the involvement of P450 mono-oxygenase enzymes in the detoxification pathway–resistance mechanism of the herbicide (53, 56).

Other P450 inhibitors that have been shown to enhance herbicide activity include the gibberellin biosynthesis inhibitors and growth regulators tetcyclasis and palcobutrazol (56) and many azol fungicides that inhibit sterol biosynthesis (i.e., triadimerol, imazalil, clotrimazol) (48). However, no P450 inhibitor that increases the herbicidal activity on a difficult-to-control weed without simultaneously increasing the phytotoxicity of the synergized herbicide on crop plants is yet known (57, 58).

Helvig and associates (59) studied the effects of lauric acid analogs as inhibitors of lauric acid and diclofop-methyl metabolism. The active lauric acid analogs showed similar profiles of inhibition of lauric acid and diclofop metabolism in wheat microsomes. In addition, the lauric acid analogs were selective since they inhibited the P450-mediated metabolism of diclofop, but not that of chlorotoluron (59).

It has long been known that insecticides can increase the activity of herbicides, and there are numerous examples of crop injury after the application of an herbicide–insecticide mixture (60, 61). Organophosphate (OP) insecticides such as malathion and terbufos have been shown to synergize the effects of a broad range of sulfonylureas and other herbicides (e.g., bentazon) by inhibiting the activity of microsomal P450s that mediate the oxidative metabolism of these herbicides (62–65). Other herbicides that

can be synergized by insecticides include diuron, linuron, chlorpropham, dicamba, pronamide, and pyrazon (66). The OP insecticides phorate, disulfoton, and terbufos have been shown to promote metribuzin phytotoxicity in soybeans when applied in combination with metribuzin (67).

## B. Hydrolytic Reactions and Toxicant Metabolism in Plants

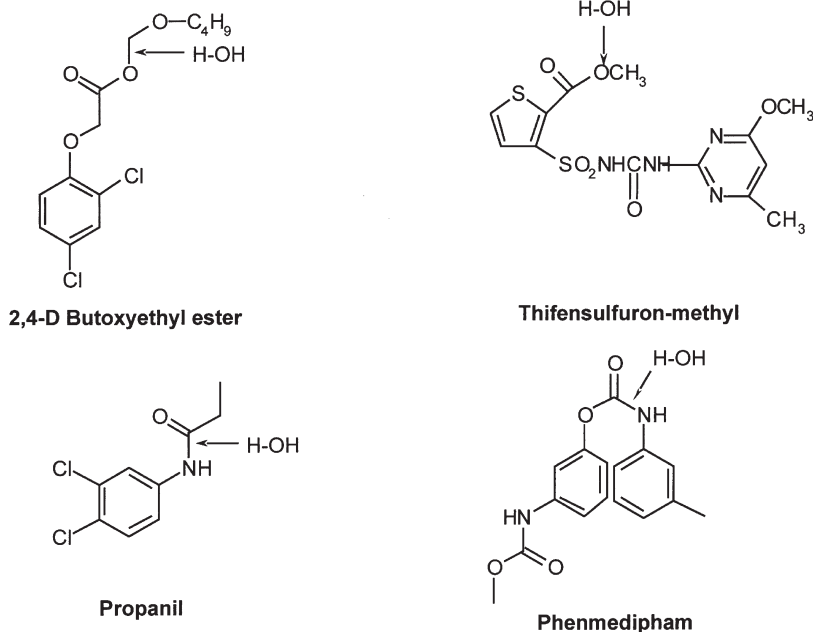
### 1. Role of Hydrolytic Enzymes in Herbicide Metabolism

Hydrolytic reactions are very important for the activation, detoxification, and selectivity of many pesticides containing amide or carbamate bonds, or esters with carbonyl, phosphoryl, or thionyl linkages (68). Many herbicides are formulated as esters to facilitate their penetration through waxy cuticles and/or to provide herbicidal activity as a result of deesterification that occurs inside plant cells (68). Herbicides activated by enzymatic hydrolytic reactions include various phenoxyalkanoate, benzoate, and aryloxyphenoxypropionate derivatives that are commonly formulated as carboxylic acid esters. For example, fenoxaprop-ethyl is rapidly hydrolyzed to the phytotoxic fenoxaprop acid in wheat and barley (69, 70).

Hydrolytic enzymes catalyze the cleavage of certain chemical bonds of a substrate by the addition of the components of water (H or OH) to each of the products. A plethora of hydrolases including amidases, esterases, lipases, nitrilases, peptidases, and phosphatases with broad or narrow substrate specificities are present in plants and other organisms (71). Thus, the potential for hydrolytic cleavage of a given xenobiotic toxicant exists in many plants. Some of the hydrolytic enzymes are cytosolic; others are associated with membranes, microsomes, and other organelles (e.g., mitochondria). The expression of the activity of hydrolytic enzymes is constitutive but may be also inducible (71).

*Ester Hydrolysis.* Esters are susceptible to hydrolysis by esterases and other enzymes (e.g., lipases and proteases). The role of plant esterases was reviewed in 1997 and in 2001 (68, 71). Plant esterases are involved in fruit ripening, abscission, cell expansion, reproduction processes, and the hydrolysis of ester-containing xenobiotics.

Short-chain aliphatic esters of phenoxyalkanoic acid and aryloxyphenoxypropionic acid herbicides (e.g., 2,4-dichlorophenoxyacetic acid [2,4-D], diclofop-methyl, fenoxaprop-ethyl, etc.) have been specifically developed as esters to facilitate absorption into treated plants and to improve selectivity. In plants, ester hydrolysis yielding the parent acid is the first enzymatic reaction, which bioactivates these herbicides.



**Figure 5** Chemical structures of herbicides hydrolyzed by plant esterase and amidase enzymes. Arrows show hydrolytic sites.

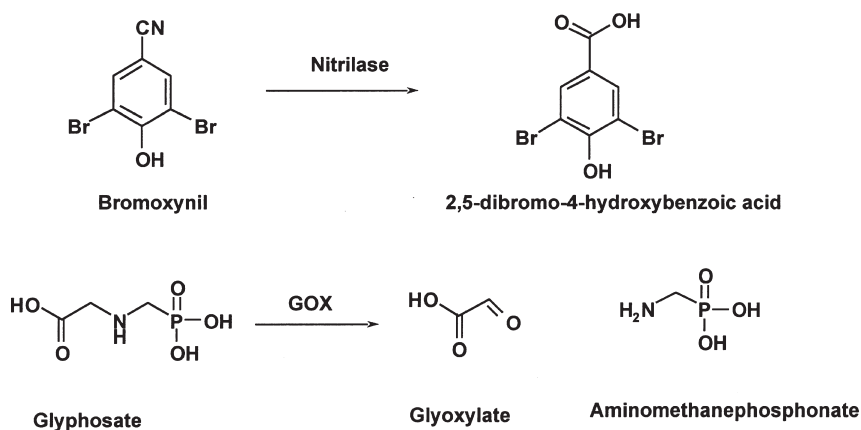
Figure 5 shows the chemical structures of herbicidal esters hydrolyzed in plants. Plant carboxylesterases capable of deesterifying herbicides such as benzoylprop-ethyl and chlorfenprop-methyl have been partially purified (72), but the physiological role of these cuticular enzymes is not known.

Other herbicides detoxified by hydrolytic reactions include some ester-containing sulfonylureas and acylamides (73). For example, the major initial metabolite of thifensulfuron-methyl in soybeans is its herbicidally inactive, deesterified free acid (74, 75). The rapid deesterification of thifensulfuron-methyl to the inactive free acid appears to be botanically specific since only soybeans among broadleaf plants are very efficient in carrying out this reaction (73). In contrast, deesterified chlorimuron acid was a minor metabolite of chlorimuron-ethyl in soybeans (74). Studies on the soil degradation of thifensulfuron-methyl have established the involvement of a microbial extracellular carboxylesterase activity, which catalyzes the deesterification of this herbicide (76). However, specific carboxylesterases catalyzing the deesterification of this herbicide in soybeans have not yet been characterized.

*Amide Hydrolysis.* Amide and substituted amide bonds are present in several classes of herbicides, e.g., acylanilides, carbamates, and phenylureas (71). Propanil is hydrolyzed in tolerant plants by an aryl acylamidase (EC 3.5.1.13) that has been partially purified from rice and some other species (77). This enzyme hydrolyzes the amide bond of propanil (Fig. 5) to form 3,4-dichloroaniline (DCA) and propionic acid (78). Barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], a troublesome weed in rice, is unable to detoxify absorbed propanil as a result of very low aryl acylamidase enzymatic activity (77). Red rice and wild rice (*Oryza*) species are capable of hydrolyzing propanil and limit the usefulness of this herbicide when they are present in fields of cultivated rice (78, 79). The continuous use of propanil in Arkansas rice production has resulted in the development of a propanil-resistant barnyardgrass biotype, capable of metabolizing the herbicide by aryl acylamidase activity (80–82). Elevated propanil metabolism mediated by an aryl acylamidase was found to be responsible for the development of resistance in jungle rice [*Echinochloa colona* (L.) Link] (83). Naproanilide, an herbicidal analog of propanil, is also hydrolyzed by rice aryl acylamidase (84).

Aryl acylamidases of several plants have been purified to homogeneity (85). The enzymes from orchard grass and rice are very similar: membrane-bound and having molecular weight of about 150 kd. These enzymes share a common pH optimum of 7.0, have similar  $K_m$  for propanil, and are inhibited by specific insecticides (e.g., carbaryl). A gene for aryl acylamidase has been cloned from male cones of Monterey pine (*Pinus radiata*) (86). The coded protein is similar to esterases containing a serine hydrolase motif in their active site and comprises 319 amino acids (86).

Apart from the limited information on the enzymological characteristics of plant esterases or other hydrolytic enzymes involved in herbicide metabolism, very little is known about the expression and regulation of genes coding for plant esterases. A gene coding for a hydrolytic enzyme deesterifying the carbamate herbicides, phenmedipham (Fig. 5) and desmedipham, has been isolated from the soil bacterium *Arthrobacter oxidans* (87). This gene has been cloned and used to engineer transgenic tobacco plants that are tolerant to these herbicides (87). Similarly, a nitrilase hydrolyzing the cyano group of the herbicide bromoxynil (Fig. 6) to the respective carboxylate has been characterized and purified from the soil bacterium *Klebsiella pneumoniae* (88, 89). Genes coding for the *Klebsiella* sp. bromoxynil nitrilase (*bxn*) have been cloned and expressed in transgenic crop plants (89–91). Transgenic cotton and potatoes tolerant to bromoxynil have been commercialized (92, 93).



**Figure 6** Enzymatic hydrolysis of the herbicides bromoxynil and glyphosate.

**C-P Bond Hydrolysis.** The most widely used phosphonate herbicide, *N*-phosphonomethylglycine or glyphosate, is completely degraded by soil microorganisms to water, carbon dioxide, and phosphate (94). However, glyphosate is metabolized rather slowly in plants, and C-P bond degrading enzymes have not been found in plants (95, 96). Mineralization proceeds without a lag phase and occurs under both aerobic and anaerobic conditions (94). Bacterial strains isolated from a glyphosate waste-stream treatment facility (97) and a municipal sewage treatment plant (98), respectively, are able to utilize glyphosate constitutively in pure culture. The breakage of the carbon-to-phosphorus bond of glyphosate has been shown to follow two main pathways (94).

In the first pathway, glyphosate (Fig. 6) is converted to aminomethanephosphonic acid and glyoxalate by a flavoprotein, glyphosate oxidoreductase (*gox*) (99). Aminomethanephosphonic acid is then directly metabolized to methylamine and orthophosphate (94) or undergoes acetylation before the cleavage of the C-P bond (94). The *gox* gene encoding glyphosate oxidoreductase was cloned from *Achromobacter* sp. (98, 100). It encodes a 46.1-kd protein with little homology to other proteins, except a region near the *N*-terminus that contains a conserved motif associated with flavin binding in flavoenzymes such as the D-amino acid oxidases or sarcosine oxidases.

In the second pathway, the initial cleavage of the C-P bond yields sarcosine, which is further converted to glycine and a C1 unit, which is incorporated into purines and some amino acids (94). The general name *C-P lyase* is used to describe enzymes responsible for the direct cleavage of organophosphonate C-P bonds (101).

## 2. Regulation of Plant Esterases and Amidases

*Safeners and Plant Hydrolytic Enzymes.* Fenchlorazole-ethyl (Fig. 3) is used as a safener to protect wheat against injury from the graminicide fenoxaprop-ethyl. The initial reaction in the plant metabolism of fenoxaprop-ethyl is its deesterification to the herbicidally active fenoxaprop acid. Crop and weed species are equally capable of carrying out this reaction. In tolerant wheat, fenoxaprop acid is metabolized further by a cleavage of the ether linkage to form 6-chloro-2,6-dihydrobenzoxazol-2-one (HOE-054014) and 4-OH-phenoxypropanoic acid (102, 103). In turn, these two moieties are further metabolized by conjugation to either glutathione (HOE 054014) or glucose (4-OH-phenoxypropanoic acid).

The safener fenchlorazole-ethyl has been shown to enhance the deesterification of fenoxaprop-ethyl to the herbicidally active fenoxaprop acid in wheat and crabgrass seedlings (104). Further metabolism and detoxification of the herbicide, however, were enhanced only in wheat, not in crabgrass. Thus, fenchlorazole-ethyl behaves as a selective safener of fenoxaprop-ethyl, protecting wheat but not crabgrass (68, 104).

In another study (105), the safener NA protected maize against injury from the sulfonylurea herbicide thifensulfuron-methyl by enhancing its deesterification. The deesterification of thifensulfuron-methyl to its parent acid, thifensulfuron, was enhanced by 30% to 50% in maize coleoptiles treated with this safener (105). Similarly, the dichloroacetamide safener furilazole has been reported to enhance the deesterification of halosulfuron-methyl to halosulfuron acid in maize seedlings by enhancing the activity of P450 mono-oxygenases with esterase activity (106).

*Synergists and Plant Hydrolytic Enzymes.* The inhibition of propanil by carbamate and organophosphate insecticides in rice is a long-established case of herbicide synergism that results from an inhibition of a hydrolytic reaction (107–110). Propanil is hydrolyzed in tolerant plants by an aryl acylamidase that has been partially purified from rice and some other species. An *in vitro* study with an enzyme extract has revealed a positive correlation between the inhibitory activities of a number of insecticides on this amidase and the phytotoxic activities of the respective insecticide–propanil combinations. It was concluded that the synergistic insecticides act by inhibiting the aryl acylamidase, which hydrolyzes propanil in tolerant plants (111, 112).

A number of carbamate (carbaryl, carbofuran), phosphate (chlorfenvinfos, phosphamidon), triphosphate (azinphos-methyl, diazinon, disyston, fensulfothion, malathion), and phosphonate (fonofos, trichlorfon) insecticides have been found to increase the phytotoxicity of propanil in rice

and/or tomato plants (107–112). Because of this interaction, rice treated with a combination of propanil and any of the aforementioned insecticides may be injured, yielding less and producing reduced dry matter. In comparative studies, carbofuran was shown to be a better inhibitor of acylamidase activity than diazinon (112).

Inhibition of an amidase is also the mechanism of the synergistic interaction between the herbicide mefenacet and the triphosphate fungicide edifenphos or several of the insecticides mentioned in the previous paragraph (113). In this case, the inhibition of mefenacet degradation appears to be more effective in the weeds *Echinochloa crus-galli* and *Echinochloa oryzicola* than in rice plants. The edifenphos–mefenacet combination can be used for improved weed control at lower herbicide doses, and with increased herbicide selectivity (113).

As mentioned earlier, when fenoxaprop-ethyl is applied to wheat in combination with the safener fenchlorazole-ethyl, the rate of fenoxaprop-ethyl deesterification and subsequent conjugation reactions is enhanced. However, when fenchlorazole-ethyl is applied with fenoxaprop-ethyl to the sensitive large crabgrass, fenoxaprop-ethyl is deesterified rapidly but does not undergo further metabolism. The greater fenoxaprop-ethyl toxicity to large crabgrass observed in the presence of fenchlorazole-ethyl is an example of indirect synergism, which results from the safener-mediated induction of the deesterification of this herbicide (104).

### C. Reductive Metabolism of Toxicants in Plants

Reductive metabolism of toxicants in plants is rather rare. The reductive deamination of the triazinone herbicides metribuzin and metamiltron is a well-documented case of reductive metabolism in plants (114). Metribuzin and metamiltron are transformed initially to their deaminated or diketo derivatives, which are then conjugated with glucose (114, 115). Picolinic acid *tert*-butylamide (PABA; MZH-2091) is one of the few xenobiotic chemicals that have been developed deliberately as selective synergists for herbicides (116). MZH-2091 (Fig. 4) enhances the weak activity of metribuzin on ivyleaf morningglory (*Ipomoea hederacea*) without affecting the tolerance of soybeans to metribuzin.

In the presence of the synergist the metabolism of metribuzin in ivyleaf morningglory is strongly inhibited, and the formation of both deaminated metribuzin and conjugates is decreased drastically. In vitro deamination of metribuzin by isolated peroxisomes was not sensitive to MZH-2091 (117). The peroxisomal deamination of metribuzin is dependent on reducing conditions, which can be met by ascorbate or glutathione.

It has been proposed that the decreased rate of metribuzin deamination in ivyleaf morningglory treated with MZH-2091 may result from a synergist-mediated depletion of the endogenous supply of a reductant, possibly ascorbate (117).

#### IV. SECONDARY METABOLISM OF TOXICANTS IN PLANTS

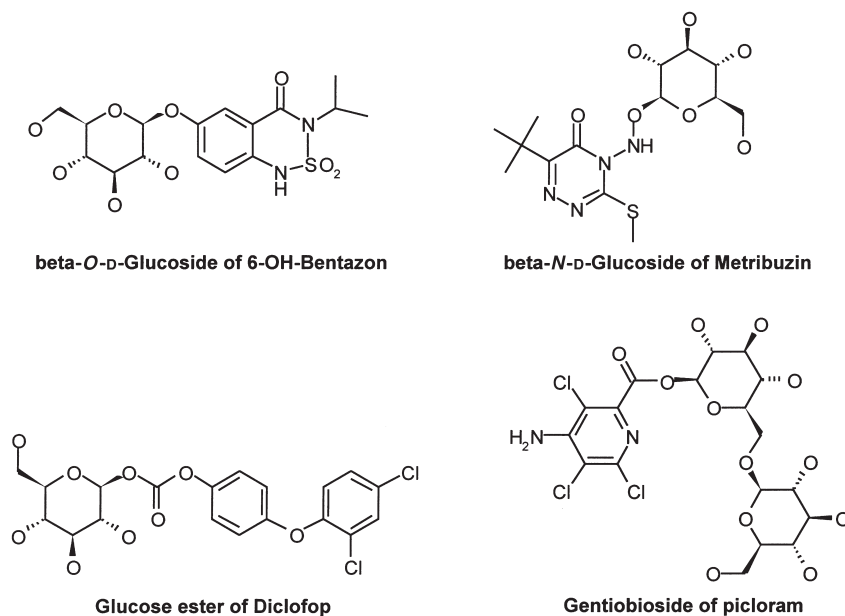
The secondary phase of toxicant metabolism in plants results in the formation of metabolites with greatly reduced or no phytotoxicity, higher water solubility, and limited mobility (15, 16). In this phase, higher plants are able to conjugate a diverse range of agrochemicals with natural plant constituents such as glucose, glutathione, and amino acids. It is becoming more and more evident that plant enzymes involved in the metabolism of secondary plant products carry out the conjugation reactions of many xenobiotics. Because of the great diversity of plant secondary metabolism, the conjugation of specific xenobiotics by certain plant species is highly variable. The recent molecular characterization of the gene–enzyme system of plant glutathione transferases has advanced our understanding of the regulation and heterologous expression of these enzymes in higher plants. The enzymological characteristics of glucosyltransferases and the molecular biological characteristics of their genes are less understood at the present time.

##### A. Sugar Conjugation of Toxicants in Plants

The conjugation of endogenous or xenobiotic toxicants with sugars is commonly described as glycosylation (118). In plants, toxicant molecules with functional groups such as  $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{SH}$ , or  $-\text{COOH}$  can conjugate with sugars such as glucose, forming *O*-, *N*-, or *S*-glucosides and glucose esters. The glucosides and/or glucose esters formed can be processed further, forming disaccharide conjugates (i.e., gentiobiosides), or undergo malonylation to form malonic acid conjugates of the glucosides (118).

Herbicides that have hydroxylated aromatic rings are conjugated mainly with glucose to form *O*-glucosides. Similarly, herbicides that contain amino groups in their molecules are known to form *N*-glucosides. Although plants are capable of conjugating endogenous substances such as flavones with galactose, glucuronic acid, and other monosaccharides, the glycosides of most xenobiotics that involve sugars other than glucose are rare (119, 120).





**Figure 7** Sugar conjugates of selected herbicides formed in plants.

The preferential involvement of glucose in the sugar conjugation of toxicants may be due to the high concentration of preexisting uridine diphosphate glucose (UDPG) in plant cells (121); UDPG functions as the immediate donor of glucose in cellulose biosynthesis (122). The majority of glucosyltransferase (GT) enzymes involved in conjugation reactions are specific for UDPG. In addition, UDPG is used as a donor for the formation of glucose esters, but in this case glucose is added to a carboxylic function (-COOH) of acidic herbicides and the reaction is reversible. Cytoplasmic esterases such as  $\beta$ -glucosidases hydrolyze glucose esters, yielding back the acid form of the toxicant (118).

O-Glucosylation has been shown to play an important role in the plant metabolism and selectivity of several classes of herbicides that are hydroxylated during the primary phase of their metabolism (123, 124). Such herbicides include phenoxyalkanoic acids (e.g., 2,4-D), benzoic acids (e.g., chloramben), picolinic acids (e.g., picloram), aryloxyphenoxypropionates (e.g., diclofop-methyl), amides (e.g., diphenamid), sulfonylureas (e.g., chlorsulfuron), imidazolinones (e.g., imazethapyr), triazinones (e.g., metribuzin), and benzothiadiazoles (bentazon) (118, 121–123, 124). Figure 7 shows the  $\beta$ -O-D-glucoside of the herbicide bentazon formed in soybean (125, 126), the N-glucoside of metribuzin formed in tomato (127), the

glucose ester of diclofop-methyl formed in wheat (128), and the disaccharide conjugate of picloram formed in leafy spurge (129).

### 1. Glucosyltransferases

The formation of most acyl-glycosides and the glycosylation of hydroxyl and amino groups are catalyzed by UDP-sugar-dependent glucosyltransferases (EC 2.4.1). Of these enzymes, the best characterized are the UDPG-dependent glucosyltransferases (GTs). The majority of plant GTs are soluble monomeric enzymes that have a molecular weight in the range of 45 kd to 55 kd, though GTs involved in sterol conjugation are membrane-associated (130).

It is now evident that plant GTs exist in multiple isoforms, each form having a discrete range of activities. Their low abundance and instability, as well as the tendency of their isoforms to copurify in chromatographic separations, have hampered the characterization of plant glucosyltransferase enzymes. Thus the *bronze 1* gene in maize, which is involved in the synthesis of anthocyanin pigments, encodes a flavonoid 3-glucosyltransferase, and the corresponding gene has also been cloned from *Antirrhinum majus* and *Petunia hybrida* (18). However, despite recent successes in characterizing GTs involved in the metabolism of natural products, relatively little is known regarding plant GT enzyme participation in xenobiotic conjugation (118).

*O-Glucosyltransferases.* *O*-glucosyltransferases (*O*-GTs) extracted from soybean cell cultures were assayed in the presence of UDPG for their ability to conjugate a range of agrochemicals (131). High activity was found to phenolic compounds such as 2,4-dichlorophenol and the 5-hydroxy- and 6-hydroxy derivatives of the herbicide bentazon; lower activities were found toward 8-hydroxybentazon (131). A 44.6-kd soluble protein with *O*-GT activity to 6-OH-bentazon was purified 115-fold from soybean cells (132). This protein also conjugated endogenous phenolic metabolites such as the flavonoid quercetin and is different from a membrane-associated 53-kd *O*-GT, which preferentially conjugated the natural product 4-hydroxyphenylpyruvic acid. Other soybean *O*-GTs, however, appear to be highly selective for xenobiotics, showing little affinity for endogenous phenolic substrates. The *O*-GTs that have activity to pentachlorophenol (PCP) were first identified from wheat and soybean cell suspensions, with molecular weight of 43 and 47 kd, respectively (133). The 47-kd PCP *O*-glucosyltransferase from soybean cells was purified more than 1000-fold and found to be highly specific for chlorinated phenols, having little activity to endogenous phenolic substrates (134).

*N-Glucosyltransferases.* *N*-glucosyltransferases (*N*-GTs) extracted from soybean cells were able to conjugate chloramben and 3,4-dichloroaniline (131). This activity appears to be identical to that of the *N*-GT identified in soybean seed, which was able to conjugate a range of arylamines (135). The *N*-GT conjugating 3,4-dichloroaniline has been partially purified from soybean cells and was assigned a molecular weight of 43 kd (134). The enzyme conjugated several chlorinated anilines but was inactive with alkylamines and several herbicides. In wheat cell cultures as well as wheat and soybeans plants *N*-GT activity has also been determined (136). Soybean cell cultures showed no *N*-GT activity to metribuzin (131, 134), but an *N*-GT conjugating metribuzin was detected in tomato cell cultures (137).

## 2. Regulation of Toxicant Glucosylation in Plants

Lamoureux and Rusness (138, 139) showed that the safener BAS-145138 (Fig. 3) partially protects maize from chlorimuron-ethyl injury by increasing the rate of herbicide metabolism by hydroxylation, glucosylation, and glutathione conjugation. Kreuz and colleagues (140) have also reported that the safener cloquintocet-mexyl enhanced the rate of hydroxylation and *O*-glucosylation reactions involved in the metabolism of the aryloxyphenoxypropionate herbicide clodinafop-propargyl in wheat. However, detailed studies on the activation of UDP-glucosyl transferases by safeners are not available.

## B. Glutathione-Mediated Metabolism of Toxicants in Plants

### 1. Role of Glutathione Conjugation in Herbicide Metabolism

Glutathione (GSH,  $\gamma$ -glutamylcysteinylglycine), found primarily in its reduced form, is the most important nonprotein plant thiol needed for the normal function of key metabolic processes such as protein synthesis and protection of plants against injury caused by active oxygen species (AOS), xenobiotics, air pollutants, and low temperatures (141–144). In addition, in 1997 glutathione was implicated as playing an important role in the regulation of gene expression in chloroplasts and other compartments of the plant cell (145). In some plant species, homoglutathione (hGSH,  $\gamma$ -glutamylcysteinyl- $\beta$ -alanine) occurs in place of GSH, and in these species hGSH conjugation occurs in a manner analogous to GSH conjugation (146, 147).

The detoxification of herbicides and other xenobiotics by glutathione conjugation depends greatly upon the levels of glutathione and the activity of specific glutathione *S*-transferase (GST) enzymes found in

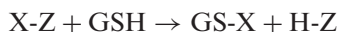
plant species. For example, the tolerance of 16 plant species to chloroacetanilide herbicides has been positively correlated with GSH or hGSH levels, and with the rate of GSH or hGSH conjugation (148). When GSH and GST levels were induced with dichloroacetamide safeners, plant resistance to these herbicides increased (149). On the other hand, when GSH in maize seedlings was depleted with buthionine-*S,R*-sulfoxamine, an inhibitor of  $\gamma$ -glutamylcysteine synthetase, the phytotoxicity of metolachlor increased (150).

In addition to chloroacetanilides, other herbicides known to conjugate with glutathione include sulfoxidized thiocarbamates (e.g., EPTC), chloro-s-triazines (e.g., atrazine), triazinone sulfoxides (e.g., metribuzin), sulfonylureas (e.g., chlorimuron-ethyl, triflurosulfuron-methyl, thifensulfuron-methyl), aryloxyphenoxypropionates (e.g., fenoxaprop-ethyl), diphenylethers (e.g., fluorodifen, acifluorfen), oxyacetamides (e.g., fluthiamide), thiodiazolidines, and sulfonamides (e.g., chloransulam-methyl) (151, 152). The chemical structures of some herbicides that are known substrates of plant GSTs are shown in Fig. 8.

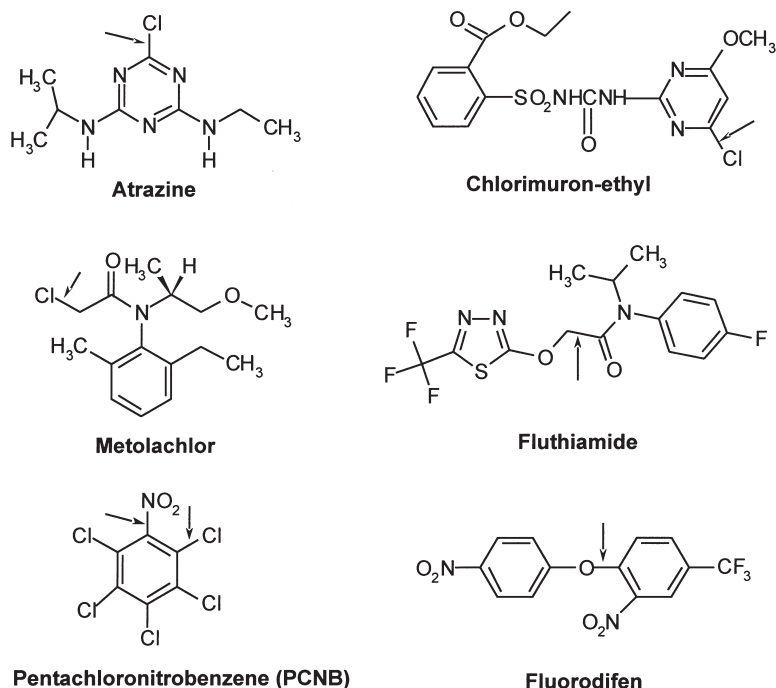
In two biotypes of velvetleaf (*Abutilon theophrasti* Medic.) from Maryland and Wisconsin 10-fold atrazine resistance has been shown to result from enhanced detoxification of atrazine via glutathione conjugation (153, 154). The mechanism of velvetleaf resistance to atrazine appears to involve an enhanced catalytic constant for GST activity utilizing atrazine as substrate (155).

## 2. Glutathione Transferases

Plant glutathione *S*-transferases (GSTs; EC 2.5.1.18) are an ancient and ubiquitous family of dimeric proteins, and their genes encode specific subunits ranging in size from 25 to 30 kd (151, 156, 157). The GST enzymes catalyze the nucleophilic attack of the thiol group of glutathione to various electrophilic substrates (123, 151, 152), according to the following equation:



The functions and regulation of plant GSTs were reviewed in 1996 and 2001 (156, 157). The GSTs are present in every type of plant tissue examined and at every stage of plant development from early embryogenesis to senescence. The GSTs have been identified in at least 59 plant species (158), and in maize, GSTs are among the most abundant nonphotosynthetic enzymes, making up as much as 1% of the soluble protein (157, 158). Although the endogenous roles of plant GSTs are still poorly understood (151, 156, 159), their involvement in cellular protection against oxidative



**Figure 8** Chemical structures of herbicides conjugating with glutathione in plants. Arrows show sites of glutathione attack.

damage and as carrier proteins for auxins has been proposed (142, 143, 156, 157). The GSTs participate in the detoxification of lipid peroxides and carbonyl derivatives produced by oxygen-reactive intermediates (ORIs) (151, 156). By analogy with the mammalian enzymes, when plant GSTs function as carrier proteins they utilize a putative second nonenzymatic binding site for the temporary storage and modulation of auxin activity (160). In addition to their suggested endogenous functions, plant GSTs play a well-established role in the metabolic detoxification of several herbicides, as discussed earlier.

Multiple GST isozymes conjugating herbicides have been characterized in maize, wheat, sorghum, and rice (151, 156, 157). At least eight GST isozymes have been characterized in maize (157). Five of them are constitutive; the other two are inducible by herbicide safeners (157). Seven GST isozymes were separated in sorghum, and two of them have been characterized (161). Among eight dimeric GSTs of wheat, three are constitutive and the other five inducible by safeners (151). Multiple GST isozymes have also been identified in rice and two of them

characterized (162–164). Using a BLAST search for GST sequences in a large database of expressed sequence tags (ESTs), McGonigle and coworkers (165) have identified 25 clones for soybean and 42 clones for maize and obtained accurate full-length sequences. Treatment with herbicide safeners resulted in an increased expression of GST activity in maize shoots (165).

The elucidation of crystal structures of three plant GSTs (one from *Arabidopsis* and two from maize) since the late 1990s has advanced our knowledge of the interactions of the active site of GSTs and their substrates at the molecular level (166–170). X-ray crystallography demonstrated that the three plant GSTs analyzed are homodimers, and that each subunit consists of two spatially distinct domains connected by a linker segment of variable length (166–170). The smaller *N*-terminal domain (domain I) is highly conserved, and specific amino acid residues facilitate the binding of GSH or maintain enzyme structure. For example, a characteristic *cis*-proline bond observed in the three plant GST structures (166–170) is crucial for the correct folding of the glutathione binding site. In addition, the *N*-terminus is responsible for the catalytic activity through activation of the GSH thiol group in four classes of GSTs. The larger *C*-terminal domain (domain II) is less conserved, is completely  $\alpha$ -helical, and consists of six amphipathic helices that form a right-handed spiral (166–170).

Each subunit contains a kinetically independent active site, composed of two distinct subsites known as the G-site and H-site. The hydrophilic and highly specific G-site is the glutathione-binding site; the adjacent hydrophobic H-site is less specific and promotes the binding of structurally diverse substrates (156, 169). The structural homology shared by plant and mammalian GSTs is significant, but they differ in the *C*-terminal region, where plants seem to have a broader and deeper H-site (156, 169). In general, amino acid residues present in the G-site are conserved or conservatively replaced, whereas variations within the H-site are more common, constituting an important part of the structural diversity of GSTs (156, 169). In 1997, Bieseler and associates (170) proposed a molecular model for the binding of the glutathione conjugate of the herbicide fluthiamide to the active site of GST of *Arabidopsis thaliana*.

### 3. Regulation of Plants Glutathione S-Transferases

Biotic or abiotic factors such as wounding, fungal pathogens, ethylene, heat shock, heavy metals, auxins, herbicide safeners, and herbicide synergists have been shown to regulate the expression of genes that code for GSTs in plants (156, 157, 171, 172). Because of the broad

nature of these modulators, it seems likely that the safener-mediated induction of herbicide-degrading enzymes may be a part of a general stress response.

*Safeners and Glutathione-Mediated Reactions.* Safeners enhance the glutathione conjugation of chloroacetanilide and sulfoxidized thiocarbamate herbicides either by elevating the levels of reduced glutathione (GSH) or by inducing the activity of glutathione-dependent enzymes. Safeners may elevate GSH levels in protected plants either directly or indirectly by (a) regulating the assimilatory sulfate reduction to cysteine, (b) activating key enzymes involved in the biosynthesis of GSH, or (c) inducing the activity of glutathione reductase (41–45).

Enhanced metabolism of chloroacetanilide and sulfoxidized thiocarbamate herbicides by GSH conjugation could also result from a safener-induced increase of the activity of the respective GST enzymes that catalyze this reaction in protected grass crops. A strong correlation between the ability of a safener to increase GST activity and its efficacy in protecting maize, grain sorghum, rice, and wheat from chloroacetanilide injury has been demonstrated (41, 43, 172). Pretreatment of maize, grain sorghum, and rice with safeners (Fig. 3) such as dichlormid, benoxacor, flurazole, fluxofenim, and fenclorim greatly enhances their low intrinsic tolerance to thiocarbamate and chloroacetanilide herbicides by inducing GST activity and in turn elevating the rate of the herbicide detoxification via GSH conjugation. The chemical structures of selected herbicide safeners are shown in Fig. 3. The safeners themselves are not toxic, but they do exhibit structural similarities to herbicides and appear to act by inducing gene expression. The exact mechanism of the safener-mediated enhancement of GST activity is not completely understood; it appears to be a stepwise process, requiring signal transduction from putative safener receptors to other cellular sites that results in the transcriptional activation of specific plant GSTs (172). In addition to the aforementioned safeners, NA and benzenesulfonamide safeners are known to induce the expression of genes that code for GSTs and other enzymes in maize (173), *Arabidopsis thaliana* (174), and wheat (175).

*Synergists and Glutathione-Mediated Reactions.* Herbicides detoxified by GSH conjugation can be synergized by the manipulation of GST activity or GSH levels. Large-seeded crop plants such as maize and soybean tend to have higher levels of GSH or hGSH in the very early seedling stage than small-seeded weeds, so synergism by depletion of GSH or GST is a possible method of herbicide synergism (176).

A successful example of this approach is the use of the herbicide tridiphan (Fig. 4) as a selective synergist of atrazine on several panicoid

grass weeds such as fall panicum (*Panicum milliaceum*), giant foxtail (*Setaria faberi*), proso millet (*Setaria italica*), and large crabgrass (*Digitaria sanguinalis*) (177–180). The basis for this synergistic interaction is inhibition of atrazine detoxification by tridiphane. Boydston and Slife (177) showed that tridiphane inhibited the metabolism of atrazine in both maize and giant foxtail, but maize was still able to metabolize the majority of atrazine in the presence of tridiphane.

Atrazine is metabolized by GSH conjugation in both maize and giant foxtail, and the selective synergism of atrazine by tridiphane in giant foxtail appears to be due to differences in the inhibition of the GST-catalyzed conjugation of atrazine (177, 178, 180). Although tridiphane mainly synergizes atrazine, synergism has also been reported for combinations of tridiphane with EPTC or alachlor in maize and fall panicum (178) and with cyanazine in giant foxtail (177). Tridiphane inhibited the in vitro GSH conjugation of pentachloronitrobenzene (PCNB), fluorodifen, and propachlor in extracts from pea and equine liver (181). In addition, tridiphane acts as a synergist of the resistant housefly by inhibiting GST activity (182).

In plants, tridiphane is converted to its tridiphane–glutathione conjugate, which appears to be the actual GST inhibitor (178). The tridiphane–GS conjugate is stable in giant foxtail, but unstable in maize. The tridiphane–GS conjugate inhibits a variety of GST enzymes with several different substrates and has been reported to be a competitive inhibitor with respect to GSH (178).

*Regulation of Glutathione S-Transferase Gene Expression.* In contrast to mammalian systems, where xenobiotic regulating elements (XREs) are found in multiple copies of GST and P-450 genes (183), plant GST promoters do not contain functional XREs or electrophile responsive elements (EpREs) (156). Instead, some of the aforementioned inducers of plant GSTs seem to work through a single element in the promoter, termed the *octopine synthase (ocs)* element (184). The *ocs* elements are 20 bp in size, identified first in promoters of genes from plant pathogens (CaMV and *Agrobacterium tumefaciens*), and are activated by wounding (184). The *ocs* elements of plant GST promoters appear to be stress-inducible elements similar to the activator protein-1 (AP-1) sites and respond to biotic and abiotic agents that generate conditions of oxidative stress (156, 184). However, it must be noted that so far, *ocs* elements have only been found in promoters of GST genes in the type III or *tau* class (171).

It is expected that multiple regulatory elements are present in the promoters of most GST genes, some of which react to specific



signals, and some of which to more general stress-related signals (171). Whereas genes of the *theta* class of plant GSTs have not been shown to contain *ocs* elements in their promoters, they are induced at the messenger ribonucleic acid (mRNA) level by herbicide and safener treatments (156). In 1999, Jepson and associates (185) proposed the existence of a safener response element (SRE), consisting of the sequence –ATTTCAA–, in the promoters of maize GSTs.

Treatment of plants with chemicals such as herbicides and safeners constitutes a type of stress and therefore elicits a signal over one of the different stress signaling chains present in plants (e.g., jasmonic acid and other plant hormones). The involvement of a common factor in the signaling transduction pathway, from the initial recognition of the stimulus to the activation of gene expression of plant GST genes, is supported by the fact that a subset of GST genes are activated by oxidative stress and the activity of the encoded proteins is needed for the cellular protection against oxidative damage (186, 187).

Stress treatments induce an oxidative burst like the one studied mostly in pathogen infections (186, 187). The active oxygen species (AOS) generated either directly or indirectly in plant-pathogen systems lead to the activation of defense genes, including GSTs, that are needed to protect the cells against oxidative damage (156). In addition, AOS stimulate phytoalexin biosynthesis and promote the hypersensitive response of pathogen-infected tissues of plants. The AOS lead to membrane damage and the generation of hydroperoxides, which may be the actual inducers of GST genes in plants (156, 171).

### C. Amino Acid Conjugation of Toxicants in Plants

Endogenous growth regulating substances (e.g., auxins) and agrochemicals that contain a free carboxyl group (–COOH) are known to form amino acid conjugates through peptide bonding (188). Phenoxyacetic acids such as 2,4-D undergo amide linkage to a variety of amino acids, mainly aspartate and glutamate, but these reactions are more prevalent in dicotyledonous plants, which are sensitive to these herbicides (189). Similar conjugates have been detected in the metabolism of the pyridinyloxyacetic acid herbicides such as triclopyr (190). Amino acid conjugation appears to be a reversible reaction and, similarly to that of glucose ester conjugation, is not regarded as an effective means of detoxification by plants (191). It is notable, however, that some amino acid conjugates of 2,4-D were compartmentalized in the plant cell vacuole, thus removing the herbicide from its possible target sites in the cytosol (192).

## V. TERTIARY METABOLISM OF TOXICANTS IN PLANTS

The glucosides and glutathione conjugates of toxicants formed in the secondary phase are further catabolized in plants, mainly through conjugation with malonic acid. In addition, glutathione conjugates are susceptible to cytosolic or vacuolar peptidases, which cleave the glyciny and glutamyl moieties from the glutathione molecule, yielding the cysteine conjugate of specific agrochemicals. Cysteine conjugates can then undergo conjugation with malonic acid.

### A. Malonyl Conjugation

Malonylation appears to provide a signal for removal of conjugates from the cytoplasm into the vacuole or across the plasma membrane (193). Furthermore, in the case of 6''-*O*-malonylglucosides, the addition of malonic acid may stabilize conjugates against cellular digestion by glycosidases (133, 194).

Malonic conjugates of *O*-glucosides have been observed in the plant metabolism of several agrochemicals, such as methazole (195), pentachlorophenol (196), flumprop (197), and 4-nitrophenol (196). Similarly, a malonylglucoside was a major product of the phenol moiety displaced from acifluorfen during the conjugation of this herbicide with homogluthathione in soybean (198). Early studies with the herbicide diphenamid (199) provided evidence for the presumptive role of malonylation in permitting the transport of glucoside conjugate into the cell vacuole.

Malonylglucosides of chloroacetanilide herbicides such as propachlor may be formed after the degradation of the putative *S*-oxide of the cysteine conjugate resulting from the homogluthathione conjugate in soybean (181). *S*-cysteine conjugates derived from the degradation of glutathione conjugates can undergo *N*-malonylation, and the malonic acid conjugates can undergo further metabolism or accumulate as significant residues. Agrochemicals that give rise to *N*-malonylcysteine conjugates include propachlor, pentachloronitrobenzene (PCNB), and EPTC (181, 200, 201).

*O*-Malonyltransferases (*O*-MATs) have been purified from chickpea (202), parsley (203, 204), and soybean (134). The enzyme from chickpea roots had a molecular weight of 112 kd and was highly specific for the 7-*O*-glucosides of two endogenous isoflavones (202). The *O*-MATs purified from parsley suspension cells conjugated the 7-*O*-glucoside of flavonoids and the 3-*O*-glucosides (203, 204). Both enzymes had a molecular weight of 50 kd. An *O*-MAT activity involved in the metabolism of pentachlorophenol (PCP) has been identified in cell suspensions of wheat and soybean (133).

The enzyme is responsible for the malonylation of the  $\beta$ -D-glucoside of PCP. The wheat enzyme was found to have a molecular weight of 43 kD; the one from soybean had 47-kD weight (133). Subsequent analysis showed that the soybean *O*-MAT copurified with the PCP *O*-glucosyltransferase, indicating that these two enzymes are closely associated (134).

*N*-Malonyltransferases (*N*-MATs) catalyze the metabolism of xenobiotics with free amino groups, mainly aniline derivatives. The resulting amide-linked malonates are turned over more slowly than malonate esters and in some plants are exported from the cells. The *N*-MATs are mainly low in abundance, but they have been purified from mung bean (205, 206) and tomato (207, 208). In tomato a 38-kD *N*-MAT appeared to be specific for conjugating 1-aminocyclopropane-1-carboxylic acid (ACC) (207), whereas amino acids such as D-tryptophan were conjugated by a 48-kD enzyme (208). A 55-kD *N*-MAT from mung bean did not conjugate xenobiotics such as 3,5-dichloroaniline (206). The role of *N*-MAT in the detoxification of anilines has been studied in soybean; a soybean 48-kD *N*-MAT with specificity for chlorinated anilines has been purified and was found to be inactive to D-tryptophan or various herbicides (209).

## B. Fate of Glutathione Conjugates

Glutathione conjugates are not persistent in plants but undergo a well-established degradation pathway initiated by stepwise peptide cleavage (210). Processing of GS conjugates is important, since the conjugates are inhibitory to GSTs (210). The complexity of the catabolism of xenobiotic glutathione conjugates in plants is exemplified by the apparent glycosylation of an intact GS conjugate of the safener benoxacor (211). In plants, GS conjugates are transported via adenosine triphosphate (ATP)-dependent tonoplast transporters into the vacuole (212–214), where carboxypeptidases acting on GS conjugates are located (215).

Plants initially remove the glycyl residue by the action of a carboxypeptidase that yields the  $\gamma$ -glutamylcysteine dipeptide conjugate (210, 215). This action contrasts with the metabolism of GS conjugates in animals, where the  $\gamma$ -glutamyl residue is removed first. After a second peptide bond cleavage, the *S*-cysteinyl derivative is formed. This can occur rapidly such that the intermediate dipeptide conjugate is not always apparent. The resulting cysteinyl conjugates may reappear in the cytosol or the apoplast as stable end products or as reactive intermediates available for further metabolism (210, 212). Thus, *S*-cysteinyl conjugates can either become *N*-malonylated, giving a fairly stable product (210), or undergo further degradation to thiolactic acid, thiophenol, and thioanisole derivatives.

*N*-malonylation of cysteine conjugates occurred in the metabolism of propachlor, EPTC, and PCNB (210). Unusually, the *S*-cysteinyl conjugate of atrazine underwent nonenzymatic rearrangement to the *N*-cysteinyl adduct in sorghum (216), which was subsequently metabolized to the *N*-lanthionine. Degradation of *S*-cysteinyl conjugates to thiolactic acids has been observed in the metabolism of several pesticides; these may then be *O*-malonylated or *O*-glucosylated (212, 213). Thioanisole (*S*-methyl) residues were identified in studies with PCNB and fluorodifen (217–219). Plant enzyme studies proved that the thioanisoles could arise from *S*-cysteinyl conjugates via the transferase activities (217, 219). In fluorodifen metabolism by spruce cells, the thiol arising from putative C-S lyase action on the *S*-cysteinyl conjugate accumulated as the *S*-glucoside (218). When the *S*-glucoside was fed back to the cells, it was then transformed to the free sulfonic acid (218).

## VI. COMPARTMENTATION OF DETOXIFIED TOXICANTS IN PLANTS

Glutathione conjugates, glucosides, or other terminal metabolites of detoxified xenobiotics may be stored as soluble metabolites in plant cell vacuoles or deposited as bound residues into biopolymers found in the cell walls of plant cells.

### A. Vacuolar Compartmentation of Detoxified Xenobiotics

#### 1. Membrane Transporters of Xenobiotic Metabolites

In 1982, Schmitt and Sandermann (220) demonstrated for the first time the vacuolar sequestration of malonylated glucosides of 2,4-D in soybean cells. The 1997 demonstration that a fluorescent *S*-bimane-glutathione conjugate accumulated in plant cell vacuoles (221) provides strong evidence that xenobiotic metabolites can be sequestered in the vacuole. The vacuolar compartmentation of detoxified xenobiotics has been the subject of numerous reviews since the early 1990s (212–214, 221–224).

The mechanisms by which conjugates of natural products and xenobiotics are transported across plant cell membranes are not well understood at the present time (223, 224). By analogy with what is known in mammalian systems, many xenobiotics and their conjugates are believed to be actively transported across plant membranes, notably the tonoplast of plant vacuoles, by ATP-binding cassette (ABC) transporters. In animals, multiple ABC transporters exist as large membrane-bound proteins

consisting of two membrane-spanning domains, each associated with an ATP-binding domain. Mammalian ABC transporters are encoded by a superfamily of genes of considerable sequence diversity, differential specificity to solutes, and sensitivity to drug inhibition.

Studies in *Arabidopsis thaliana* have confirmed the presence of multiple genes that encode ABC transporters present in plants, but their functions and specificities have not been fully characterized (225). Glutathione conjugate transporters (described also as GS-X pumps) are present in the tonoplast membrane, and plant vacuoles appear to serve as a site for the temporary storage of glutathione conjugates of xenobiotic and endogenous substances (226–229). The GS conjugate transporters contain a region that undergoes phosphorylation (P-domain), a site that recognizes GSH (G-domain), and a site with affinity to the electrophilic moiety of the glutathione conjugates (C-domain) (156, 226–228). Plant GS conjugate transporters are MgATP-dependent, are characteristically specific for S-linked GS conjugates or oxidized glutathione, show no activity toward reduced glutathione or its component amino acids, and are sensitive to inhibition by vanadate (156, 226–229).

In barley vacuoles, the MgATP-dependent uptake of the GS conjugates of *N*-ethylmaleimide and the herbicide metolachlor was demonstrated (226). This transporter appeared to be similar to one involved in the uptake of oxidized glutathione (228), but distinct from one transporting the bile acid taurocholate (221). In mung bean vacuoles the transporter involved in the uptake of the GS conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) was studied in detail. Uptake of the conjugate was inhibited by the GS conjugate of metolachlor, suggesting that the transporter was also involved in herbicide metabolism (228). Similar transporters were also identified in vacuoles isolated from *A. thaliana*, beetroot, and maize (228). Although the identity of the GS conjugate transporter has not been demonstrated unequivocally, when the *AtMRP1* gene from *A. thaliana* was expressed in yeast, it catalyzed the uptake of GS-CDNB, GS-metolachlor, and oxidized glutathione (230). The sequence of *AtMRP1* resembled closely that of the *HmMRP1* gene, which encodes a GS conjugate transporter involved in multiple drug resistance in humans, and the *ScYCF1* gene in yeast, which confers tolerance to cadmium. Jamai and colleagues (231) demonstrated active uptake of oxidized glutathione by broad bean protoplasts and suggested that ABC transporters may be present in plasma membranes too. This uptake was inhibited by GS conjugates.

Investigations on the active uptake of glucosylated xenobiotics into plant vacuoles are rather limited. Galliard and coworkers (232) showed that the uptake of hydroxyprimisulfuron glucoside into barley vacuoles

was MgATP-dependent, but independent of the transport of the GS–metolachlor conjugate.

## 2. Regulation of Vacuolar Transporters

The vacuolar transport of xenobiotic conjugates is modulated by xenobiotic treatments. The uptake of the GS–CDNB conjugate by vacuoles of mung bean was enhanced by CDBN treatments in a concentration-dependent fashion (233). The safener benoxacor antagonized the effects of CDBN on this system (233). Similarly, the vacuolar transport of the GS–metolachlor and hydroxyprimisulfuron–glucoside conjugates in barley was increased after treatment with the safener cloquintocetmexyl, whereas treatment with the safeners benoxacor and naphthalic anhydride resulted in marginal increase (232).

Although the mechanisms of the safener-mediated enhancement of transporter activity have not been determined, kinetic analysis suggested that increased uptake was due to the presence of an increased number of transporters in the vacuolar membrane (233). Evidence that the expression of vacuolar transporters in response to xenobiotic treatments is transcriptionally regulated has been found in *A. thaliana* (225). The sequences of four ABC transporters characterized from *A. thaliana* resembled those of ABC transporters present in humans and fungi. Treatment with various xenobiotics enhanced the expression of mRNA transcripts coding for three of these four ABC transporters. One of these, termed *EST2*, was elevated more than 40-fold by certain compounds (225).

## B. Bound Residues of Toxicants in Plants

In addition to the partitioning of soluble conjugates through cellular membranes, the bioavailability of xenobiotic conjugates may be reduced by their association with components of the lignified plant cell wall. Plant metabolic studies with radiolabeled xenobiotics usually document a “bound” residue fraction that is nonextractable with common aqueous and organic solvents. Nonextractable or bound residues of pesticides in plants have been discussed in older and more recent reviews (5, 234–236).

The incorporation of agrochemicals and their primary metabolites into nonextractable residues is well documented (5, 234–236). Agrochemicals metabolized through the glutathione conjugation pathway can give rise to residues that become significantly incorporated into the nonextractable plant fraction (237). In the case of PCNB metabolism in peanut this process is believed to occur via a thiophenol catabolite (237) incorporated into lignin (238). These bound residues appear to be more

abundant in plant roots than in cell cultures, the latter forming preferentially the long-lived *N*-malonylcysteine derivative (237). By contrast, catabolism of the cysteine conjugate in roots occurred via a presumptive C-S lyase activity leading to the thiophenol (237). Thus, caution should be exercised when metabolism of agrochemicals is studied in plant cell cultures rather intact plants (239).

Sugar conjugates can also be apparent intermediates in the incorporation of agrochemical residues into cell wall components (240). However, there is scarce information on the dynamic relationship between sugar conjugates and their deposition in the cell wall. It is possible that sugar conjugates can bind directly to cell wall constituents, as has been proposed for the *O*-glucoside of a natural product (240). Alternatively, release of the aglycone may occur as a prelude to incorporation. Laurent and Scalla (241) reported in 1999 that nonextractable residues of phenoxyacetic acid and its hydroxylated (4-OH) metabolite were associated with hemicellulose and lignin of soybean cell suspension cultures. Similarly, Schmidt and colleagues (242) showed that a xenobiotic *O*-gentiobioside formed in a cell culture system was not a terminal residue, but degraded to the corresponding glucoside and to nonextractable residues. This notably occurred in the late stages of cell culture, characterized by increased cell lignification (242).

Studies on the potential effects of safeners on the formation of secondary conjugates of herbicide metabolites are scarce. The dichloroacetamide safener BAS-145138 did not cause any significant alterations in the formation of bound residues and soluble secondary metabolites from the initial glutathione conjugates of the chloroacetanilide herbicides propachlor and metolachlor in maize (138, 139).

## VII. CONCLUSIONS

The metabolism of agrochemical toxicants in plants is a major factor that contributes to their development and safe and efficient utilization. The metabolism of a given agrochemical in plants is a multistep process that includes oxidative, reductive, hydrolytic, and conjugation reactions as well as vacuolar compartmentation and extracellular deposition processes. Our current understanding of the metabolism and elimination of toxicants in plants has been facilitated by rapid advances in analytical and organic chemistry, molecular genetics, and immunology during the past two decades.

Studies related to the identification of xenobiotic metabolites in plants, characterization of plant gene–enzyme systems detoxifying xenobiotics, and



their regulation by biotic and abiotic factors will continue to draw the attention of academic and industrial researchers in the future. Advances in analytical methods used to detect and characterize metabolites of xenobiotics in plants and soils will continue to facilitate the registration of commercialized pesticides and increase our understanding of the metabolism and elimination of plant toxicants.

Nevertheless, major breakthroughs in this field will result from the rapid advances in our understanding of the molecular genetics of the enzymatic systems detoxifying xenobiotic toxicants in higher plants and microorganisms. The recent completion of the genome sequences of *A. thaliana* and rice and the future analysis of the genome sequences of other important crop plants will provide us with greater opportunities for identifying and characterizing plant enzymes involved in the stepwise process of the metabolism of toxicants in plants. The availability of these enzymes for heterologous recombination studies will assist the prediction of the metabolism and biological activity of agrochemicals and their metabolites in plants. Furthermore, plant genomics and DNA microarray techniques will facilitate the screening of new compounds being developed by computational chemistry as herbicides or as modulators of herbicide activity (i.e., safeners and synergists).

Coding sequences of the gene-enzyme systems that detoxify agrochemicals in plants will also facilitate the development of transgenic plants with modified tolerance to commercialized herbicides. The feasibility of this approach has already been demonstrated with the use of microbial genes to genetically engineer transgenic crops for resistance to several herbicides, such as bromoxynil, desmedipham, glufosinate, and glyphosate. Better insights into the relationships between the metabolism of endogenous substances and xenobiotic toxicants will facilitate our understanding of the principles underlying the species-dependent selectivity of many commercialized agrochemicals.

In the final analysis, the utilization of the technologies described for the development of new selective agrochemicals and new transgenic plants for use in agricultural production must be evaluated in terms of the sustainability of agroecosystems and their profitability for farmers and food producers around the world.

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# 10

## Host–Pathogen Relations: Diseases Caused by Viruses, Subviral Organisms, and Phytoplasmas

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In this chapter the biochemical and physiological events leading to damage and disease symptoms in virus-infected plants are briefly summarized. Naturally, this summary is by far not complete.

Although virus diseases of plants have been known a long time, discovery of plant viruses and plant virology itself are only about 100 years old. As an introduction we have to summarize some definitions and terms.

### I. VIRUSES

*Viruses* are submicroscopic, infectious nucleoproteins, obligate intracellular parasites. The most important features of viruses are as follows:

Virus particles are produced from the assembly of preformed components.

Virus particles (*virions*) themselves do not grow or undergo division.

Viruses lack the genetic information for the generation of metabolic energy or for protein synthesis (no metabolism).

Viruses have only either infectious ribonucleic acid (RNA) or deoxyribonucleic acid (DNA).

Until recently, one of the basic tenets was that plant viruses, unlike bacterial and animal viruses, did not integrate into host genomes. Data over

the past few years have broken this tenet, and now an increasing number of integrated plant viral sequences are being found in plant genomes (1).

The damage caused by virus infection is manifested on plants by various *symptoms*. In the case of compatible interaction that generally means systemic spread of the virus without cellular disruption. On the other hand, infection of plants by viruses results in reduction of plant size and yield. Since most plant virus infections are not accompanied by clear damage (in contrast to infection by animal viruses), the resulting yield reduction remains an enigma. A better understanding of the biochemical and physiological background of plant–virus interactions should help to explain yield reduction caused by virus infection.

Plant viruses are normally unable to initiate infection without assistance; they cannot penetrate the plant cell wall. According to recent knowledge, no plant virus has a specific receptor, as do animal and bacterial viruses, to attach to cells. Therefore, plant viruses need mechanical damage of the cell wall in order to introduce virus particles into the plant cell, by a *vector* associated with transmission of the virus or by *mechanical injury* of cells. After successful replication in an initial cell, plant viruses need a special mechanism to infect new cells. Plant cell walls contain channels called *plasmodesmata*, which allow plant cells to communicate with each other and to pass metabolites between them. Since these channels are too small to allow the passage of virus particles or genomic nucleic acids, most plant viruses encode for specialized *movement proteins* that modify the plasmodesmata. One of the best known examples of this is the 30-kD protein of tobacco mosaic virus (TMV).

### A. Generalized Scheme of Virus Replication and Assembly

Most plant viruses have a *positive (+) sense single-stranded (ss) RNA genome*. This type of genome is ready to function as a messenger RNA (mRNA) after entry into a host cell; therefore, it is usually infectious.

The genome of *negative (–) sense ssRNA* viruses must be copied into (+) sense mRNA in order to be infectious and functional in virus replication. A viral-encoded enzyme (transcriptase) carries out this copying.

Viruses with a *double-stranded (ds) RNA genome* also use transcriptase to copy genomic RNA into mRNA.

Plant viruses with *ds or ssDNA genomes* use different host enzymes to produce functional mRNA.

In a generalized scheme of plant virus replication virus particles (virions) enter the host cell and disassemble: i.e., the nucleic acid (RNA) of the virus is freed from the protein coat. The infected cell is induced

to synthesize viral RNA polymerase. This enzyme utilizes the viral RNA as a template and forms complementary RNA, which is a mirror image (complementary copy) of the template viral RNA. The mirror image (–) strand then serves as a template for more virus (+ strand) RNA synthesis.

### 1. Assembly of New Virus Particles

As soon as the new viral nucleic acid is produced, some of it is translated: i.e., the host cell is induced to synthesize proteins encoded by the viral genome. These proteins are used by the virus as a coat or in other functions (e.g., replication, movement). Finally, the new viral nucleic acid and coat protein subunits are assembled together to form new virus particles (virions).

## B. Symptoms of Virus Diseases

Most virus names were derived from the symptoms they cause on host plants. A plant that does not support virus replication or translocation is called *resistant*. Susceptible plants allow infection and movement of viruses. Usually a *combination of several symptom types* shows up in a virus-infected plant (Fig. 1).

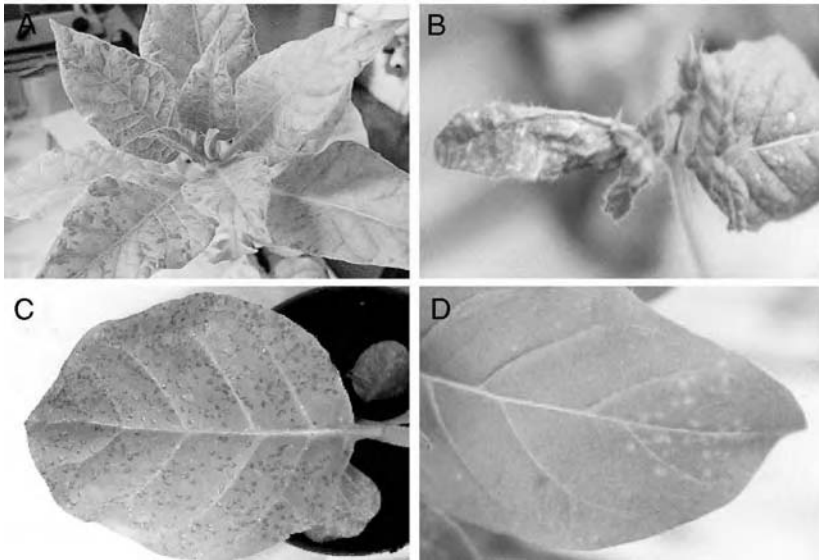
A virus can multiply to a very high concentration and yet induce *no obvious symptoms* in an infected plant. Infection with a mild strain of the virus in a tolerant host results in a symptomless infection. *Localized symptoms* usually develop near the site of entry on the leaves, fruit, or other organs and are the result of a resistant reaction of the host plant. Most types of symptoms are *chlorotic* (living cells) or *necrotic* (dead cells) *lesions* and *ringspots*, ranging from single rings to concentric rings of a chlorotic or necrotic nature. *Systemic symptoms* are the results of systemic invasion of the host plant by a virus. When viruses reach the vascular tissues, they are transported throughout the plant and cause systemic infection. Depending on the appearance of symptoms they can be classified as follows:

*Mosaic patterns* are more common symptoms in virus infection.

*Mosaic* means an alternating pattern of tissues with two different colors. If the borders between areas are sharp, then the symptom is called *mosaic*; if they are diffuse, it is called *mottle*.

Infection by some viruses may induce *yellowing* throughout the infected plants. Viruses that cause this type of symptoms are usually phloem-limited.

*Stunting and dwarfing* of the infected plants are additional common symptoms of virus infections. The degree of dwarfing varies. As a



**Figure 1** Some characteristic symptoms of virus diseases. A and B, systemic symptoms in susceptible plants. C and D, localized symptoms in resistant plants. A, systemic mosaic in tobacco (*Nicotiana tabacum* cv. Samsun nn) infected with the U1 strain of *Tobacco mosaic virus* (TMV). B, systemic necrosis in *Nicotiana clelandii* infected with *Cauliflower mosaic virus* (CaMV) strain W260. C, necrotic lesions (hypersensitive reaction) on a tobacco (cv. Xanthi nc) leaf inoculated with TMV strain U1. D, chlorotic lesions on a *Nicotiana clelandii* leaf inoculated with CaMV strain W260.

rule, the size of the infected plants is almost always smaller than that of a healthy one. Interference with plant growth hormone levels is regarded as the most important factor for dwarfing. Other factors include reduced photosynthesis and reduced translocation of nutrients.

When an entire virus-infected plant becomes necrotic, the result is called *systemic necrosis*. *Malformation* means change of the normal shape of a plant or parts of a plant.

### C. Metabolic Changes

Symptom expression is determined by the viral genes or their translation products. It seems reasonable to believe that the interaction between RNA sequences or their translation products and the host plant results in different symptoms. However, the exact mechanisms are still unknown.

In the following paragraphs effects of viral infection on plant metabolism or distinct metabolic pathways and biochemical or molecular events leading to development of symptoms are discussed (2, 3). Recently, caution was urged in the explanation of experiments measuring changes of host metabolism after virus infection, because, the researchers claimed, there are many variable factors (4).

### 1. Photosynthesis

The most common symptoms of systemic virus infections are mosaic pattern, chlorosis, mottling, and retarded growth of the host plant. Generally, virus infection reduces photosynthesis, but our knowledge of the exact mechanisms is limited. A reduced number of chloroplasts, decreased chlorophyll content, and various ultrastructural and biosynthetic alterations have been described (5, 6). Symptoms related to the inhibition of several processes in chlorophyll biosynthesis, delay or inhibition of plastid development, and formation of photosynthetic apparatus in barley seedlings infected with *Barley stripe mosaic virus* (BSMV) were also reported in 2002 (7, 8).

Some viruses, such as *Turnip yellow mosaic virus* (TYMV), induce obvious clumping and fragmentation of the chloroplasts and formation of large vesicles. It is noteworthy that viral capsid proteins of TMV and *Cucumber mosaic virus* (CMV) have been reported to enter chloroplasts.

Altered chloroplasts can be responsible for chlorosis and retarded growth of infected plants. Reduction of chloroplast numbers and abnormalities of chloroplasts in virus-infected tissues have been commonly observed by several researchers. Disorganization or impaired development of lamellae, membrane vesiculation, increase in the size of plastoglobuli, cytoplasmic invagination, and accumulation of starch grains are common symptoms. Generally, chloroplast changes closely resemble those that occur during senescence. There is little evidence for the involvement of plastids in virus replication. The chlorosis of the virus-infected tissues is accompanied by a reduction in chlorophyll content. The reduction in chlorophyll content may be associated with the enhanced activity of chlorophyllase, but this reduction may be a consequence, not the cause, of chloroplast damage. In some cases there is a reduction in chlorophyll synthesis; however, in other instances, e.g., in tobacco infected with *Tobacco etch virus* (TEV), there is no change in chlorophyll content although the number of chloroplasts decreases.

The photochemical activity is usually reduced, often apparently more than can be accounted for by the reduction in chlorophyll content. Chloroplasts from virus-infected plants may reduce the Hill reaction activity by 50%. Photophosphorylation is also reduced; however, the decrease in photochemical activity may only begin late after infection or may remain unaffected.

CO<sub>2</sub> assimilation is reduced in many infected tissues, but in some virus diseases of plants there is no difference between the healthy and infected plants. Doke and Hirai (9, 10) showed long ago that in tobacco infected with TMV or *Potato virus X* (PVX) there was an early stimulation of CO<sub>2</sub> assimilation in infected cells and around the site of infection. After several days of infection the CO<sub>2</sub> assimilation declined even in that case.

Inhibition of starch synthesis is a common feature of virus infection. However, both starch synthesis, and starch degradation may be affected. There may be a proliferation or enlargement of starch grains in chloroplasts of chlorotic and necrotic tissues due to limited transport caused by blockage of plasmodesmata. A general feature of virus infection is that the products of carbon fixation (sugars) are directed into pathways that lead to the production of nucleic acids and proteins (2, 4).

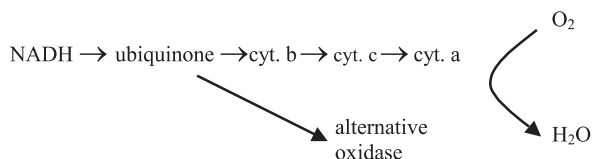
*Mosaic symptoms* can be very heterogeneous with respect to virus infection, just as yellowing, mottle, and mosaic symptoms are obviously due to a reduction of leaf pigments. Carotene and xanthophyll give the yellowish coloration as a result of a loss of chlorophylls, although the previous pigments are also decreased in some diseases. This effect is probably due to cooxidation with unsaturated fatty acids released from thylakoid membranes by phospholipases. In addition, reduction in carbon fixation is one of the most commonly reported effects in leaves that show mosaic or yellows diseases.

It was found recently that dark green islands in the mosaic symptom that are free of virus are caused by *posttranscriptional gene silencing* (see later discussion).

## 2. Respiration

In host-virus combinations associated with systemic virus spread and with mosaic or other nonnecrotic symptoms, the *respiration rate* slightly increases. However, in the chronically infected host the rate of respiration may be lower than normal. In necrotic hosts with the hypersensitive response to virus infection, there is a much more pronounced increase in the respiratory rate. These changes were either coincident with the appearance of local necrotic lesions or appeared before symptom expression. It remains to be determined whether the increase in the rate of respiration is connected with the stimulation in mitochondrial number in the virus-infected local lesion host or is the result of enhanced activity of polyphenol oxidase or peroxidase enzymes. The *in vivo* function of these latter enzymes seems to be unimportant in the *respiratory pathways*, however.

The increase of respiration rate may result from the uncoupling of oxidative phosphorylation from respiration. It has also been shown that



**Figure 2** The alternative pathway of respiration. NADH, reduced nicotinamide adenine dinucleotide; cyt., cytochrome.

in a virus-infected local lesion host the pentose phosphate pathway is stimulated and the enzymes of the *Embden–Meyerhof–Parnas* glycolytic pathway remain unchanged. This finding has been confirmed in several cases by a couple of investigators (2, 11). On the other hand, in systemic hosts the shift to the pentose phosphate cycle does not occur or is expressed only slightly. Interestingly, the cyanid-resistant or alternative pathway of respiration (see Fig. 2) has also been involved in the local lesion type of response to viral infections by restricting hypersensitive response (HR)-type cell death as a result of low generation of reactive oxygen species (ROS; see later discussion).

Alternative respiration does not couple with adenosine triphosphate (ATP) synthesis and relates to energy dissipation. This type of respiration is characteristic of tissues in which respiration rises dramatically, as in the virus-infected local lesion hosts. Alternative oxidase lowers the generation of ROS in the mitochondria by preventing overreduction of the electron transport chain (12), thereby restricting the size of necrotized lesions. Ordog et al. (13) have shown that the alternative oxidase plays a role in restriction of HR-type cell death but not HR-associated viral resistance.

### 3. Secondary Metabolism

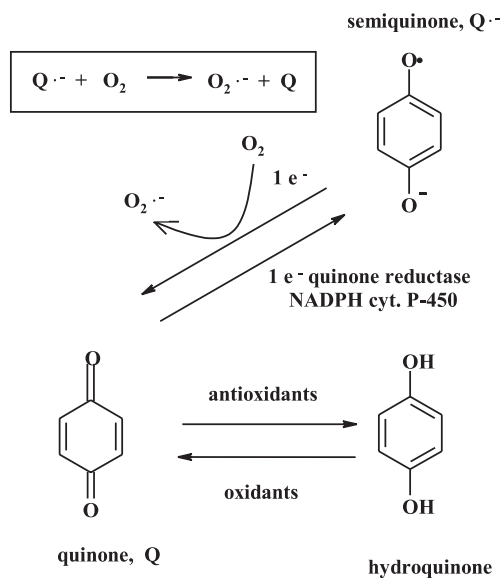
As regards secondary metabolism, again we have to distinguish between necrotic symptoms and nonnecrotic symptoms caused by virus infections, the latter associated with mosaic and yellow discoloration. The most important changes in diseased plants are connected with alterations in phenol compounds, their oxidation products, as well as enzymes involved in phenol biosynthesis and oxidation.

*Systemic Infection.* There is little change in phenol content as well as in the activity of peroxidase and polyphenoloxidase after TMV infection. Local lesion-forming tobacco races containing the *N* gene, when held at or above 30°C, become systemically infected. Under these conditions phenylalanine ammonia lyase (PAL) activity may be below that of uninfected plants. Correspondingly, levels of several phenolics, such as chlorogenic acid, rutin, and caffeoyl-quinic acid, also decrease after infection (14).



*Local Lesion Formation (Hypersensitive Response).* As a rule, the hypersensitive response (HR) of the local lesion host plant is accompanied by increase in the activity of polyphenoloxidase in several host-virus combinations. Oxidized phenols probably do not have a direct causal relationship with necrotization in plant tissues. Reducing compounds (antioxidants) such as ascorbic acid, glutathione, and cysteine, which keep oxidized phenolics in a reduced, and therefore in a less toxic, state, were able to suppress necrotic lesion formation in a local lesion host of virus infection (15). It was established that this type of antioxidant may inhibit the harmful effects of ROS, which are claimed to cause damage to plants. One can suppose that antioxidants suppress formation of semiquinone, which is one of the sources of production of superoxide and other ROS (Fig. 3).

Accumulation of phenolics in and around the necrotized lesions of virus-infected local lesion hosts is a well-established fact. Chlorogenic acid and scopoletin were observed by Legrand and colleagues (16) and by Goy and coworkers. (17). Activity of PAL is stimulated in local lesion hosts before appearance of visible necroses. Interestingly, a stimulated activity of PAL was also observed in cells at the lesion periphery that were beyond the necrotized area. Inhibition of PAL activity pointed to the possibility that



**Figure 3** Antioxidants suppress the formation of quinone and semiquinone and production of superoxide anion ( $O_2^{\cdot -}$ ). NADPH, reduced nicotinamide adenine dinucleotide phosphate; cyt., cytochrome.

this important enzyme may have a role in restriction of multiplication of viruses (18; see Sec. I.C.3).

In short, virus infection leads to stimulation of enzymes of phenol biosynthesis and oxidation of phenolics. This process is more intensive when the host reacts with an HR (necrotization). However, it remains to be seen whether accumulation of phenolic compounds and oxidation of phenolics are cause or consequence of tissue necrotization during the hypersensitive response.

#### 4. Translocation

A number of phloem-limited viruses induce general yellowing of the entire plant, although these viruses are not distributed throughout the mesophyll and parenchyma cells. In these cases virus infection probably interferes with the nutrient uptake and movement of nutrients from one part of plants to another (see Sec. I.C.1, for the discussion of starch grains).

Some effects of virus infection, such as necrosis, which reduces the efficiency of phloem tissues, must limit translocation of fixed carbon from mature leaves to growing tissues. In addition, a reduced permeability of leaf cells to the migration of sugars into the phloem is a limiting factor.

It is noteworthy that the rapid progress in studying the molecular mechanisms of systemic spread of plant-infecting viruses revealed an interaction between virus movement and macromolecular trafficking in plant tissues (4).

#### 5. Growth Regulators

Changes in the metabolism of growth regulators caused by virus infection often appear as visible symptoms such as stimulated or inhibited growth, hypoplasia, hyperplasia, tumors, leaf flecks, and epinasty. On the other hand, changes in the endogenous hormonal levels or external application of growth regulators have also influenced the plant–virus interaction or the symptoms, which are discussed later.

There are many publications reporting on changes of cytokinin, auxin, ethylene, or abscisic acid levels (2, 4). Usually auxin level is reduced, but a substantial increase in auxin activity associated with severe symptoms has also been reported. External application of auxin to plants may inhibit virus replication.

Gibberellin concentrations are often reduced after virus infection, whereas those of abscisic acid are increased. Interestingly cytokinin activity may be either reduced or increased. Stimulation of ethylene production is associated with necrotic or chlorotic symptoms, generally with stress (see later discussion).

Virus infections, as a rule, influence the synthesis, degradation, or translocation of plant growth hormones (19). In addition, different hormones may interact with each other, and their concentrations may be altered with the age of the plant. Thus, hormonal changes usually contribute substantially to the symptoms of virus-infected host plants (mosaic, yellowing, necrosis, epinasty, abscission).

*Hyperauxiny* or *reduced auxin levels* may result from virus-infection. In bean plants infected with *Southern bean mosaic virus* (SBMV) axillary buds develop most probably as a result of loss of apical dominance controlled by indoleacetic acid. This and other symptoms of virus-infected plants were amended by treatment with indoleacetic acid, naphthalene acetic acid, or indole butyric acid. Van Loon and Berbé (20) have shown that in a local lesion tobacco host the concentration of auxin substantially increased during the necrotic lesion enlargement. However, in systemic hosts (with mosaic symptoms) a marked decrease of auxin concentration was observed (21).

Exogenous application of different auxins may affect virus replication, but the action depends on several factors. In protoplasts of a tobacco variety that reacts systemically to TMV, multiplication of the virus was reduced; however, in protoplasts from two hypersensitive hosts it was stimulated after auxin application (22).

Some enzymes that can reduce the level of auxin *in vivo* (peroxidases, indoleacetic acid oxidase) could be the cause of stunting caused by virus infection (23). The situation is also influenced by some phenolics, such as coumarin and scopoletin, that may stimulate or inhibit the oxidation of indoleacetic acid under natural conditions.

Depression of *gibberellin* level in several virus-infected plants has been associated with stunting of diseased plants. Exogenous application of gibberellic acid again was able to reverse, partially at least, the stunting symptom of infected plants (24, 25).

The *cytokinin* level seems to be enhanced after infection in both systemic and local lesion hosts (26). External application of this hormone to the infected plant exerts variable effects, depending on whether it is applied before or after infection. Treatments may influence both virus multiplication or only counteract the formation of necrotic spots (27, 28). Balázs and associates (29) have shown that in Xanthi-nc tobacco, which is a local lesion host of TMV, the total number of infection sites was not reduced; only the number of visible necrotic spots was suppressed in cytokinin-treated tobacco plants. Many visible single-cell necroses were seen by Evans blue staining in the kinetin-treated tissues. Sziráki and colleagues (30) found in Xanthi-nc leaves in which the systemic acquired resistance was induced higher cytokinin level. In this case the lower leaves of tobacco

plants were infected with TMV or *Cucumber mosaic virus* (CMV) as well as with other necrosis-inducing compounds, and in the upper leaves higher cytokinin level and fewer visible necrotic lesions were detected upon challenge infection. Cytokinin may cause inhibited necrotization in the upper challenge-inoculated leaves, which had systemic acquired resistance.

*Ethylene* production is stimulated in hosts, which produce necrotic lesions upon infection with the virus; however, in systemic hosts the level of ethylene remains unchanged (31–33). In chlorotic lesions caused by *Barley yellow mosaic virus* (BYMV) in *Tetragonia expansa* or in CMV-infected cucumber ethylene production accompanied the chlorotic symptom development. Interestingly, exposure of infected plants to ethylene stimulated necrotic spots in the chlorotic area (34, 35). However, CO<sub>2</sub>, an inhibitor of ethylene action, inhibited development of necrotic lesions. Ethylene production after virus infection was also associated with epinasty and elongation of the hypocotyl (36).

*Abscisic acid* level seems to be increased substantially in most of the virus-infected plants that develop necrotic lesions (HR) upon infection or react with mosaic symptoms in systemic hosts (34, 37). If a local lesion host of TMV was treated with abscisic acid before infection, an increase in local lesion number was experienced. Accumulation of virus particles and synthesis of viral RNA were stimulated in systemic hosts (37, 38). Thus, it would seem that this hormone influences establishment of infection and accumulation of the virus in the host plant differently. Abscisic acid may inhibit cell division and, therefore, could be associated with the observed inhibition of leaf growth of virus-infected plants. This hormone may act in concert with ethylene and other plant growth regulators; therefore, its different roles in symptom expression remain unresolved (39).

## 6. The Oxidative Burst and the Role of Antioxidants

Many pathogenic and abiotic stresses are accompanied by rapid production of reactive oxygen species (ROS), causing damage of plant tissues (40–45). It is also known that during senescence formation of reactive oxygen species and lipoxygenase enzyme activity are increasing and enzymatic and nonenzymatic antioxidant capacity are decreasing in plants (46).

It was shown (47, 48) that in the TMV-infected *N* gene-containing local lesion tobacco host a superoxide (O<sub>2</sub><sup>•−</sup>) generating system is induced. However, this is not the case with a systemic host lacking the *N* gene. If the local lesion tobacco host was held at higher than 28°C neither were local lesions developed nor was O<sub>2</sub><sup>•−</sup> generation enhanced. Thus, ROS were associated only with formation of necrotic symptoms and were absent when virus multiplication and spread were accompanied by nonnecrotic

symptoms. Furthermore, in a transgenic local lesion host (NahG tobacco) that produces large necrotic lesions upon TMV infection, an increased level of  $O_2^{\bullet-}$  production was found at the edge of necrotic spots, as compared to that in the control nontransgenic tobacco (49).

It is known that activation of ROS associated with infections and stresses is counteracted by up-regulation of antioxidant defense systems. This is true even in tobacco plants in which systemic acquired resistance (SAR) was induced. In TMV-infected Xanthi-nc tobacco (a local lesion host) both the ROS and the antioxidants were activated, not only in the infected leaves but also in the noninfected upper leaves of the same plant (50, 51). However, in the transgenic line of the same tobacco (NahG) in which the SAR was inhibited, the antioxidants were down-regulated (49).

Thus, it was hoped that in breeding plants with high antioxidant capacity, resistance to necrotic symptoms caused by viral and other infections could be created (52). Indeed, it was shown earlier (53, 54) that *in vitro* selection of tobacco tolerant against paraquat (a ROS-producing herbicide) produces plants with a high antioxidant capacity that can also tolerate tobacco necrosis virus (TNV) infection, in addition to many stresses and infections associated with necrotic symptoms. The *in vitro* selected paraquat-tolerant plants that had high antioxidant capacity exhibited signs of juvenility, such as slow senescence, green color, and high phospholipid/sterol ratio in the membranes. All of these signs of juvenility were associated with the suppression of necrotic symptoms on infection with TNV and necrotrophic pathogens. Similarly, in leaves of a cytokinin-overproducing transgenic line of tobacco where senescence was inhibited and the level of antioxidants kept high, development of necrotic symptoms caused by TNV or other biotic and abiotic stresses was suppressed. Leaf disks from the cytokinin-overproducing tobacco line evolved less ethylene and substantially less ethane (an indicator of lipid peroxidation) as a result of decreased number of local lesions caused by TNV infection. This was a sign of a lower rate of stress and lipid peroxidation. In the transgenic plants, as compared to the control nontransgenic SR1 tobacco. When the antioxidant capacity of leaves was increased by treatment with propylgallate (a free radical scavenger), the number of local lesions caused by TMV infection was suppressed by more than 50%. Parallel to the lower rate of tissue injury, ethylene production, as an indicator of stress, and ethane production, as an indicator of lipid peroxidation, decreased in the propylgallate-treated virus-infected leaves (Barna et al. unpublished results).

The antioxidant capacity of plants can also be improved indirectly. Hippeli and Elstner (55) reported that the transition metal ion-catalyzed oxygen activation has an important role in pathogenic processes. Deák and

associates (56) created transgenic tobacco lines in which the ferritin gene was expressed on a high level. The protein ferritin (the gene product) is able to bind and store free iron in these transgenic plants. The free iron is necessary for the formation of the most toxic  $\text{OH}^\bullet$  (hydroxyl radical) during the Fenton reaction:  $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\bullet$ . The low level of  $\text{OH}^\bullet$  in the transgenic tobacco hindered the formation of necrotic spots caused by TNV and other pathogens as well as by paraquat itself. The so-called ferritin transgenic plants turn to be multiresistant to several stresses including viral infection (see also Chap. 2).

#### D. Hypersensitivity and Host Cell Death

As mentioned earlier, localized necrotizations are usually results of plants' resistant reactions to virus infection. Resistance is generally (but not always!) manifested as the *hypersensitive response* (HR), which causes rapid death of cells at the site of infection. The hypersensitive response is a typical programmed cell death and is induced not only in resistant plant–virus, but also in plant–bacteria and plant–fungi interactions with characteristic events such as the following:

- Rapid change of membrane potential and increase of extracellular pH
- Accumulation of reactive oxygen species
- Synthesis of new proteins, the pathogenesis-related (PR) proteins
- Increase in the production of cell wall phenolics
- Production of ethylene and phytoalexins
- Accumulation of salicylic acid

*Necrotization* itself is a symptom of incompatibility and does not necessarily inhibit the spread of the virus, since in many cases virus particles can be found outside the lesions. On the other hand, many factors such as environmental conditions or the physiological state of plants, influence development of necroses.

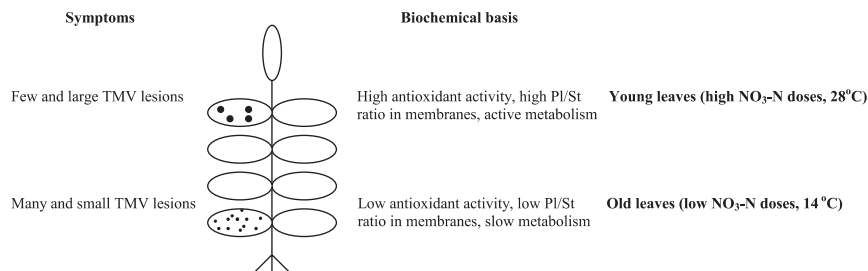
*Environmental factors such as temperature, light, or the physiological state of plants* may alter latency as well as incubation periods and the type of symptoms. A typical effect of temperature on symptoms occurs in the case of TMV and tobacco containing *N* gene. At normal temperature (below 30°C) the virus causes necrotic lesions and cannot spread; however above 30°C, TMV causes systemic infection without lesion formation (see earlier discussion). But these systemically infected plants, when restored to normal temperature, develop necrosis throughout the whole plant, resulting in the collapse and death of the plant.

The *age* of plants may have a strong influence on their sensitivity to pathogen infections and various stresses. Plants are generally more susceptible to diseases at the seedling stage than at the later stages. When late resistance to certain pathogens is unusually strong, it is called *adult plant resistance*. Although this type of resistance has great practical importance, there are very few data in the literature regarding the mechanism of adult plant resistance.

Another aging process, the *senescence* of leaves, exerts different actions on disease and stress resistance. Generally senescence of plant tissues is favorable for necrotrophic, juvenility for biotrophic pathogens. The reason for this difference is that biotrophs prefer living cells with active metabolism, whereas necrotrophs need the disintegration of plant tissues as a source of nutrients for their development. It was also found that the lower antioxidant capacity of senescent tissues provides a less effective defense against the damage produced by the oxidative burst during biotic and abiotic stresses.

When the development of necrosis on tobacco leaves of various ages was studied, lower (oldest) leaves of Xanthi-nc tobacco showed many more lesions than middle or upper (youngest) leaves upon TMV infection, and lesion diameter slightly increased from the bottom to the top leaves. The low number and the large size of TMV lesions on youngest leaves, in addition to the large number and small size of lesions on oldest leaves, indicate that lesion number is increased by senescence, but lesion diameter is governed by the active metabolism of juvenile tissues. Similarly, when enhanced metabolic activity was produced by high doses of NO<sub>3</sub> nitrogen or elevated temperature (28°C), the lesion number was strongly reduced, but the lesion size increased on TMV-infected Xanthi-nc tobacco (57, 58) (Fig. 4). In addition, in young leaves more single-cell necrotic reactions (which could be observed only microscopically) were produced, and the infectious virus content was similar to that of the oldest leaves, indicating greater virus multiplication in juvenile tissues.

In agreement with the preceding data, if senescence of leaves was inhibited by external cytokinin treatment, the number of lesions was reduced, but infectivity of 100 lesions from the kinetin-treated half-leaves exceeded that of 100 lesions from the control halves. In addition, more necrotic single cells were seen by Evans blue staining on the kinetin-treated half-leaves (29). These results again suggest that juvenility of host tissues favors virus multiplication but inhibits development of local necrotic lesions. This double effect, at least partly, explains contradictory data in the literature on the influence of cytokinin treatment on plant-virus interaction, which also depends on the mode of application (see earlier discussion). Juvenility and resistance to viral necrotization could be



**Figure 4** The effect of senescence, nitrogen doses, or temperature on TMV lesions on tobacco leaves and its biochemical basis. TMV, tobacco mosaic virus; PI/St, phospholipid/sterol.

improved by high doses of nitrogen fertilizer or by removal of the terminal bud as well (57, 59).

## E. Resistance of the Virus-Infected Plant

The aspects of plant resistance to virus infections were summarized in 2000 by Fraser (3). We report here on the most important experimental results.

### 1. Gene-for-Gene Resistance

Over the past two decades several laboratories conducted research on the interactions between host resistance genes (*R* genes) and avirulence genes (*Avr*) of pathogens. It is supposed that host *R* genes encode receptors that interact with elicitors produced by the pathogen *Avr* genes. The result is a recognition of the pathogen by the host plant. Recognition initiates a signal transduction cascade that includes salicylic acid, ethylene, or perhaps jasmonic acid (60). As a consequence, accumulation of reactive oxygen species, nitric oxide, pathogenesis-related proteins, salicylic acid, etc., has been demonstrated.

It is important to note that uncoating of virus particles readily occurs in both nonhost and host plant species. Thus, it would seem that host resistance does not influence uncoating.

According to Bendahmane et al. (61), the receptor–elicitor interaction in the virus-infected plant depends on the timing, the level of the two components, and the affinity between the elicitor and its receptor.

In the case of *extreme resistance* affinity between the two components is very high and production of elicitor occurs early after infection. This is the case in the interaction between the *Rx* gene product of resistant potato and the coat protein of the virus (PVX) that is the elicitor. This resistance is



not associated with a hypersensitive response (HR): in other words, there is no cell death (necrosis) in infected resistant tissues but a rapid arrest of *Potato virus X* (PVX) occurs. *Rx* resistance operates also in protoplasts of the host by suppressing accumulation of PVX. Interestingly, *Rx* is similar to *R* genes that determine an HR. In spite of that, extreme resistance is independent of HR. However, when the coat protein of PVX was expressed constitutively in *Rx* gene transgenic *Nicotiana benthamiana*, there was a necrotic symptom expression. On the contrary, under natural infection, when the elicitor is expressed from the viral genome, expression of the HR does not occur. It would seem that extreme resistance is epistatic to HR. Whether or not this early response of the host induces an oxidative burst remains to be seen. An additional example of *extreme resistance* is activated in tomato infected by TMV in which the responsible *R* gene is *Tm-1*. This type of resistance is also expressed in protoplasts in contrast to the HR type of resistance.

The *HR-associated resistance* occurs in *Nicotiana tabacum* carrying the *N* gene, which derives from *N. glutinosa*. This resistance is effective against TMV infection. Further examples: the *N* gene-carrying *Nicotiana* plants, the *L* gene-carrying *Capsicum* plants, and the *Tm-2* gene-carrying tomato have HR-type resistance to TMV. In solanaceous plants the interaction of *R* genes with *gene VI* of *Cauliflower mosaic virus* (CaMV), the first virus gene identified that determines avirulence (62), also results in resistance characterized by necrotic lesions (HR) or chlorotic spots. In both cases virus particles are restricted to the region immediately surrounding the necrotic or chlorotic spots.

In the case of *N* gene-mediated HR-associated resistance production of the elicitor (coat protein of TMV) occurs rather late; recognition and arrest of viral replication by the activated host resistance are also late (61). Thus, there is enough time for activation of a secondary resistance response: expression of the HR (necrosis). This HR-associated resistance is not so early as is seen in the case of extreme resistance and is related to an intermediate host response to viral infection. The viral elicitor supposedly has a lower affinity for the *R* gene-mediated receptor. This type of resistance is not expressed in isolated protoplasts, however, it is a tissue-mediated resistance phenomenon. The HR that appears in *N* gene tobacco is overcome at high temperatures (63) and above 28°C the virus intensively replicates and moves systemically. However, if infected plants are moved to low temperature (20–25°C), very intensive necrotization of tissues (HR) occurs. We have shown (Hafez et al. unpublished) that necrotization can be induced even at 30°C if we add compounds that produce ROS to the infected tobacco leaves. This happens only in combination with TMV infection because the ROS-producing compounds alone cannot

induce necrotization in healthy plants at this high temperature. The production of HR-type necrosis was inhibited or suppressed by the application of superoxide dismutase (SOD) and catalase to infected leaves at both 20°C and 30°C. It would seem that the HR-type necrosis is associated with the presence or accumulation of ROS. Interestingly, the amount of superoxide was substantially reduced at 30°C in healthy as well as in virus-infected leaves and activity of SOD was enhanced. It is also noteworthy that the HR-associated programmed cell death can be inhibited in transgenic tobaccos that carry cell death suppressor genes that derive from animals (64).

Since the late 1990s it has been shown by four research groups that virus resistance and HR are controlled separately in different host–virus combinations (61, 65–67). It turned out that extreme as well as HR-type resistance to viral infections uncouples from HR (cell death symptom). Thus, HR is only incidental to resistance, as shown earlier for plant–fungus interactions (68). It is still an unresolved problem whether production of oxygen free radicals or other ROS is required for virus resistance (47) or whether there is a defect in plasmodesmatal function of the hypersensitively resistant plant, that accounts for restriction of viral spread (69). The role of antiviral compounds isolated from resistant tobaccos also remains to be established (70, 71).

*Systemic necrosis (systemic HR)* may occur in virus-infected plants that demonstrate a late response to viral infections (72–74). The virus in this case is not limited to local necrotic or chlorotic lesions but systemically extends, causing widespread necrosis. This is a weak type of host response when resistance is activated too late during pathogenesis.

In summary, resistance is activated rapidly during extreme resistance therefore, HR-type necrosis cannot develop, but an HR would be produced if resistance were activated somewhat later and systemic HR occurs if resistance can develop rather late in pathogenesis.

## 2. Additional Mechanisms of Virus Resistance:

*Cultivar Resistance of Cowpea to Cowpea Mosaic Virus.* Ponz et al. (75, 76) have shown that a special host cultivar contains a single gene that is responsible for resistance of the cultivar Arlington to the *Cowpea mosaic virus* (CPMV). In extracts of the host protoplasts three inhibitors were determined: an inhibitor of the translation of CPMV RNA, an inhibitor of proteinase that degrades the virus proteins, and an inhibitor of proteolytic processing of virus polyprotein. It has been concluded that the proteinase inhibitor is responsible for virus resistance and the single gene is host-coded.

*Inhibition of Systemic Movement of the Virus.* In potato and corn, resistance to *Potato leafroll virus* (PLRV) and *Maize dwarf mosaic virus* (MDMV), respectively, is caused by impairing virus movement through the vascular system (77, 78). The exact mechanism of resistance is unknown at present.

*Virus Inhibitors in Plants.* Extracts of several plants can inhibit virus infections when mixed with the inoculum. One of the best known inhibitors is the 29-kD protein from *Phytolacca americana* (79).

Resistance induced by *satellite RNAs* and *defective interfering RNAs* is also a characteristic of this group (discussed later).

### 3. Acquired Resistance

Acquired resistance is associated with (the *N* gene-determined) necrotic local lesions. After appearance of the tissue necrosis (HR), the plant exhibits local or systemic resistance to a second infection.

Ross (80) has shown that in an *N* gene-carrying tobacco that developed necrotic spots upon TMV infection resistance to a subsequent infection was developed locally around a very small zone surrounding the necrotic lesions. The phenomenon was termed as *local acquired resistance*. This type of resistance, however, was not found in plants grown at high temperature (30°C), which developed no lesions after TMV infection.

In addition, Ross (81) has shown that *systemic acquired resistance* (SAR) developed in tobacco and bean plants if one leaf of the local lesion host was inoculated with the virus and remote leaves on the same plant were inoculated a week later. The SAR was expressed as a reduction in the size and number of virus-induced local necrotic lesions in remote leaves. In plants held at high temperature (30°C) the SAR did not develop. Biochemical events leading to SAR result in accumulation of salicylic acid (SA) both in the first infected leaves and later in the remote uninfected ones (82, 83). Exogenous application of SA induced the same set of genes as found in the biologically induced SAR (84). Salicylic acid is crucial for the development of SAR, although other mobile signals seem to be responsible for the systemic action. In 2002, it was suggested that a lipid transfer protein may signal the biochemical changes in the plant parts exhibiting SAR (85). Transgenic tobacco and *Arabidopsis* plants expressing the salicylic hydroxylase gene, whose product converts SA to catechol, do not to develop SAR. In addition, in this transgenic tobacco susceptibility to necrotization was increased (86).

It is noteworthy that Doke et al. (87) as well as Alvarez et al. (88) emphasized the role of a systemic microoxidative burst in the development

of SAR in plant–fungus and plant–bacterium interactions, respectively. Later it was demonstrated by electron paramagnetic resonance spectroscopy that in remote leaves of Xanthi-nc tobacco virus-induced systemic resistance is also associated with a slight oxidative burst (51).

It has been known for a long time that antioxidants suppressed TMV-induced necrotization in tobacco leaves (89). Since then several observations referred to the function of plant antioxidants in resistance (52, 90). As regards the manifold action of salicylic acid, Fodor and coworkers (50) have shown that SA may play a primary role in up-regulating active antioxidative defense. Essentially, SAR can be regarded as a resistance to the necrotic symptoms and does not necessarily arrest virus multiplication (30). It turned out that down-regulation of the antioxidant defense in a transgenic (*NahG*) tobacco, which does not develop SAR, could be the cause of its inability to reduce TMV-induced necrotic lesion formation after an attempted induction of SAR (49). In summary, SA may play a primary role in maintaining active antioxidant defense to detoxify ROS, thereby inducing development of SAR.

## F. Pathogen-Derived Resistance and Gene Silencing

The majority of plant pathogenic viruses contain a single-stranded RNA genome with genes encoding *proteins necessary for virus replication and movement and the coat protein*. In the mid 1980s, the idea emerged that expressing one or more virus genes and/or proteins in a susceptible plant host will result in resistance to the given virus; the concept became known as *pathogen-derived resistance* (91). The feasibility of this approach was later demonstrated by creating virus-resistant transgenic plants that express various forms of coat protein genes, replicase genes, and other virus-derived sequences.

Resistance to infection, manifested as a delay in the onset of symptoms, was first shown with transgenic expression of capsid protein of TMV, followed quickly by demonstrations involving the capsid protein of *Alfalfa mosaic virus* (AMV) and PVX. Later both defective and nondefective replicase genes were shown to exhibit a very strong, but specific resistance. On the other hand, expression of defective movement protein genes has been shown to provide resistance not only to closely related but to other viruses as well (for reviews see Refs. 92 and 93).

Initially it was thought that pathogen-derived resistance operates only through expression of the transgene-derived protein, but later it turned out that untranslatable viral transgenes are also able to confer resistance (94–96). This type of virus resistance, *based on the presence of transgene*

*mRNA*, rather than protein, which became known as RNA-mediated virus resistance, has the following characteristics:

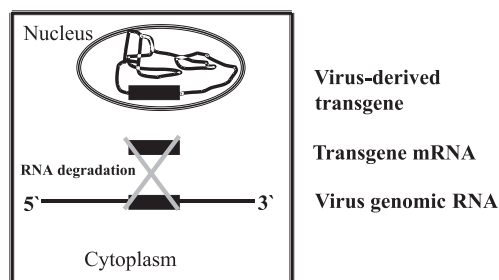
It is a *high level of virus resistance* that is not overcome by virus inoculum concentrations orders of magnitude greater than the concentrations that routinely infect wild-type plants (97).

The resistance has high *sequence specificity*: it is effective only against viruses closely related to the virus that was the source of the transgene sequence. For example, a transgene based on the coat protein of *tobacco etch virus* (TEV) confers resistance to TEV but not to other potyviruses (98). In fact, RNA-mediated resistance is effective only against viruses that have a high sequence homology (at least 80%) to the transgene (99). The requirement of such high sequence specificity (in other words, a narrow resistance spectrum) is certainly a practical disadvantage of this type of resistance.

Transgenic plants carrying untranslatable forms of viral genes sometimes display a delayed resistance phenotype (*recovery*): e.g., tobaccos transformed with the truncated coat protein gene of TEV are initially susceptible to TEV infection but plants later recover from the infection in ca. 3–5 weeks. Recovered tissue or protoplasts from it cannot be reinfected with TEV. Furthermore, a significant (five- to eight-fold) decline in steady-state levels of transgene mRNA occurs in recovered tissue. On the other hand, nuclear “run-on” studies reveal no differences in transgene transcription rates, suggesting a posttranscriptional suppression of transgene mRNA levels (98, 100).

These and other similar results imply that during RNA-mediated virus resistance a *sequence-specific RNA degradation mechanism* that *affects both the mRNA of the transgene and the replicating viral RNA* is triggered in infected plants. Interestingly, it turned out that a similar RNA degradation mechanism called *post-transcriptional gene silencing* (PTGS) is triggered in transgenic plants in which transformation with homologs of endogenous plant genes leads cosuppression of both the transgene and endogenous gene expression (101, 102) (Fig. 5).

In plants PTGS is effective against both transgenes and RNA viruses; that effect implies that it is targeted against the “foreign” sense (+ strand) RNA. It has been suggested that the mechanism operates via antisense (- strand) RNA (103), which could be produced by plant- and/or virus-encoded RNA-dependent RNA polymerases (RdRPs). Indeed, an RdRP gene that is necessary for gene silencing was isolated from *Arabidopsis thaliana* (104, 105), and a similar gene identified in tobacco plays a role in limiting virus replication and symptom development during

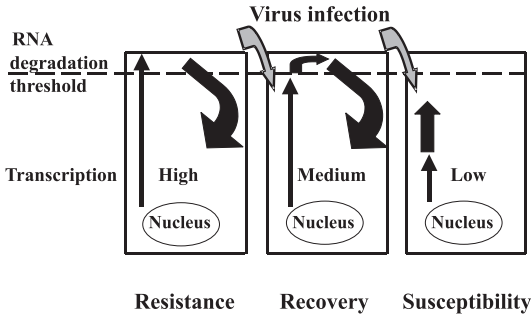


**Figure 5** Ribonucleic acid (RNA)-mediated virus resistance operates through posttranscriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism triggered in infected plants that affects both the messenger RNA (mRNA) of the transgene and the replicating homologous viral RNA. In cells of a transgenic plant, a virus-derived transgene is incorporated into the plant genome within the nucleus. Expression of the transgene results in production of an mRNA sequence that is transported into the cytoplasm. In virus-infected plant cells sequence homology between the transgene mRNA and the virus genomic RNA (homologous regions depicted as black boxes) must be at least 80% in order to initiate an RNA degradation process (PTGS) that results in elimination of the transgene and virus RNAs and establishment of RNA-mediated virus resistance.

infection by TMV and PVX (106). The final result of RdRP activity in plants is *double stranded (ds) RNA*, which appears to play a *central role in the gene silencing process*. Double-stranded RNA and transgene constructs that produce transcripts that can become double-stranded can induce strong PTGS and virus resistance in plants (107, 108). Plants that display PTGS or are virus-infected contain small (ca. 25 nucleotides long) RNA fragments homologous to the transgene or virus in both (+ and –) polarities, suggesting that these are degradation products of ds RNA(109–111).

Genes that function in the RNA degradation process have been isolated from *A. thaliana* plants (112, 113). On the basis of these and other related observations *molecular events leading to PTGS in plants* can be summarized as follows:

- Recognition by the host plant of transgene and/or virus RNA as “foreign”
- Synthesis of dsRNA using transgene and/or viral RNA as a template
- Degradation of dsRNA and all other homologous (transgene, virus) RNAs

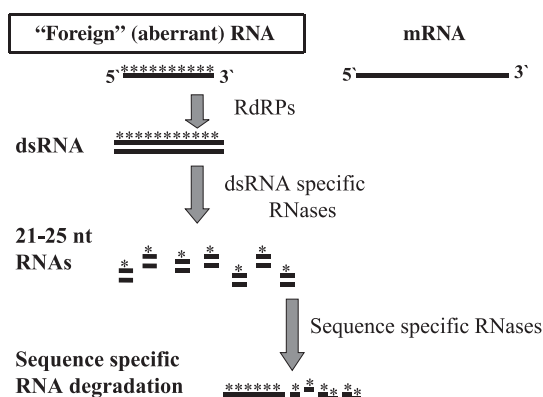


**Figure 6** The *threshold, or quantitative model* of plant posttranscriptional gene silencing (PTGS) as a possible mechanism of ribonucleic acid (RNA)-mediated virus resistance: the plant is somehow able to sense transgene and virus RNA levels and, if the level is too high, to target these RNAs for degradation. Transgenic tobacco expressing an untranslatable coat protein gene of *Tobacco etch virus* (TEV) displayed three different phenotypes on TEV infection: resistance, recovery (delayed resistance), and susceptibility. Resistant plants contained at least three integrated transgene copies, whereas susceptible and recovered plants had only one or two copies. Presence of more copies of the transgene in resistant plants produces more messenger RNA (mRNA) (higher transcription rate); the level exceeds the threshold tolerated by the plant and triggers a sequence-specific RNA degradation mechanism (PTGS). This process eliminates both transgene and, during infection, homologous virus RNAs. The medium transcription rate of the transgene in plants showing a recovery only lifts the mRNA level above the degradation threshold together with homologous virus RNAs. Susceptible plants, however, produce a low amount of transgene mRNA that, even when combined with virus RNA, is not sufficient to reach the threshold of RNA degradation and virus resistance induction. (Adapted from Ref. 134.).

One can consider plant PTGS as an immune reaction specifically targeted against genes and viruses. If so, then on what basis does the host plant consider a transgene or virus RNA “foreign”? Two currently accepted models attempt to answer this question:

(1) The so-called threshold, or quantitative model (Fig. 6) proposes that the plant is somehow able to sense transgene and virus RNA level and, if the level is too high, to target these RNAs for degradation. The hypothesis arose from work on transgenic tobacco plants that contain an untranslatable TEV coat protein gene (98, 114). The efficiency of virus resistance was related to the number of integrated copies of the transgene. One to two copies resulted only in a recovery (delayed resistance) phenotype in which the low levels of transgene mRNA would initially not be sufficient to induce resistance. However, the RNA of the replicating virus combined with the transgene





**Figure 7** The *aberrant ribonucleic acid (RNA)*, or *qualitative model* of plant posttranscriptional gene silencing (PTGS): the trigger for PTGS induction is some qualitative feature (aberrancy) of foreign, e.g., transgene or virus RNA. \*, Foreign RNA would be distinct from normal (self) mRNA because of a shorter than normal messenger RNA (mRNA), absence of a protein product, or other unusual features. As a result of such aberrancies, foreign RNA is destined for degradation through the production of double-stranded (ds) RNA (by plant- or virus-encoded RNA-dependent RNA polymerases [RdRPs]) and short, 21- to 25-nucleotide (nt) RNAs (by dsRNA-specific ribonucleases [RNases]). Finally, all other RNAs homologous to the foreign sequence are degraded by sequence-specific RNases. On the other hand, normal mRNA is not exposed to the degradation process and remains intact.

mRNA would be sufficient to reach the critical threshold necessary to induce the RNA degradation mechanism (PTGS) and virus resistance.

According to the so-called aberrant RNA, or qualitative model (Fig. 7) the trigger for PTGS induction is some qualitative feature (aberrancy) of the RNA (115). Aberrant RNA derived from either a transgene or a virus would be distinct from normal mRNA because of

- Shorter than normal transgene mRNA (premature transcript termination due to methylation of the transgene) (116, 117)

- Absence of a protein product (e.g., an untranslatable transgene used to elicit RNA-mediated virus resistance)

- Presence of complementary sequences that result in dsRNA formation (in the case of RNA viruses formation of dsRNA is an integral part of the replication cycle)

Because it is unlikely that PTGS in plants has evolved solely for the purpose of transgene suppression, so it is perhaps not surprising that some *natural virus defense* systems have been found to resemble gene silencing.



In other words, it seems that *viruses can trigger PTGS in the apparent absence of homologous nuclear (transgene) sequences*. For example, infection of *Nicotiana clevelandii* by *Tomato blackring virus* (TBRV) strain W22 results in an initial symptomatic phase in which the virus moves systemically, followed by a recovery state in which new tissue that develops post inoculation is asymptomatic and virus-free. Furthermore, silencing of viral RNA is active in the recovered tissue, conferring effective resistance to superinfection by W22 and a closely related strain, but not by unrelated strains (118). In a second example, recovery from infection by CaMV was studied in kohlrabi (*Brassica oleracea* var. *gongyloides*) (119) and oilseed rape (*B. napus*) (120). Again, recovery was closely associated with the specific loss of viral RNA independently of the presence of homologous plant sequences. The discovery that the natural viral defense systems mentioned resemble PTGS is highly significant as it indicates that gene silencing may be a general antiviral response in plants. An important unresolved issue, however, is the extent to which viruses, other than those mentioned, elicit PTGS upon infection. Most compatible virus–host combinations do not show the recovery phenotype. It is possible, however, that silencing is triggered transiently to limit the extent of virus replication and symptoms in infected tissue but is not sufficient to limit systemic spread of the virus. This possibility is supported by the observation that in virus infections with mosaic symptoms the presence of virus- and symptom-free green islands within mosaic tissues is the result of PTGS induction (121). Similarly, in PVX-infected *Nicotiana benthamiana* PTGS is induced in spite of a successful systemic virus infection (122). The possibility that PTGS is a naturally occurring antiviral response in different virus–host combinations certainly deserves careful attention in the future.

Obviously, if PTGS is a normal defense system against “foreign” nucleic acids in plants, *viruses need to suppress this defense in order to establish an infection successfully*. The first indications of this mechanism were from studies of viral synergy, in which joint infection with two viruses leads to a strong enhancement of symptoms. For example, infection of plants with various potyviruses increases the virulence of several unrelated viruses. The agent that causes this synergy is a multifunctional (aphid transmission factor and proteinase) potyviral protein, the so-called helper component proteinase (HC-Pro). The *HC-Pro* sequence of the potyvirus TEV expressed in transgenic tobacco is sufficient to promote a synergistic infection with three other unrelated viruses, PVX, CMV, and TMV (123, 124). Furthermore, the presence of HC-Pro in the plant is able to suppress silencing (PTGS) of transgenes, in other words, restore their expression (125–127). Several other virus proteins that both *promote virulence and*

*suppress PTGS* have been identified. Some of the more important *virus-encoded suppressors of PTGS* are the following:

- Potyvirus HC-Pro protein (other functions: aphid transmission factor, proteinase)
- Cucumovirus 2b protein (other functions: host-specific systemic virus movement)
- Potexvirus p25 protein (other functions: cell-to-cell movement, RNA helicase)
- Tombusvirus p19 protein (other functions: symptom determinant)

Investigation of more than 15 viruses has shown that suppression of PTGS is a widespread characteristic of plant viruses (110, 111, 126, 128). The future identification of additional virus suppressors of PTGS should help to create more insight into the mechanism of posttranscriptional gene silencing, a naturally occurring, RNA-mediated virus resistance in plants.

## II. SUBVIRAL AGENTS

Subviral agents are viroids, virusoids, satellite RNAs, satellite viruses, and defective interfering RNAs (129).

*Viroids* are self-replicating pathogens that consist only of unencapsidated, single-stranded circular RNAs of 250–400 nucleotides. Viroids do not code for any protein products including capsid protein. The structure of viroids is extremely stable, and some of them have ribozyme activity (self-cleavage). Viroids replicate in the nucleus, mainly within the nucleoli. Since viroids are not translated, their effects on plants must be a consequence of direct interaction of the viroid RNA with the host cell. Though the mechanisms of viroid pathogenesis are still unknown, the severity of symptoms seems to be the result of complex interactions among three of the five viroid domains. The pathogenicity domain is especially interesting.

*Satellite RNAs* are small RNA molecules that require a host virus for replication and encapsidation; they can be considered as parasites of plant viruses. Their size ranges from 200 to 1600 nucleotides. A representative of this subviral group is the CMV-associated RNA (CARNA 5). Larger satellite RNAs may encode a protein. The mechanism of satellite replication is poorly understood, similarly to the replication of their helper viruses.

They may or may not reduce the titer of the host virus. Generally the suppression of the replication of the helper virus genome reduces the disease symptoms, but in some cases the satellite RNAs improve the pathogenicity of the helper virus. (130)

*Virusoids* are viroidlike satellite RNAs. These small satellite RNAs have a circular, highly base-paired structure like that of a viroid. They do not encode any protein and have ribozyme activity. Virusoids depend on a host virus for replication and encapsidation.

*Defective Interfering (DI) RNAs* are small RNA molecules derived from viral RNA by extensive deletion; they depend on the original virus for replication. Defective interfering RNAs usually reduce the replication of the host virus (see 131, 132).

### III. PHYTOPLASMAS

Many plant diseases once thought to be caused by viruses are now known to be caused by phytoplasmas. Phytoplasmas are very small (200–500 nm) phloem-limited plant pathogenic bacteriallike prokaryotes that lack a cell wall. They belong to the prokaryotes *mycoplasmas*, which require cholesterol for membrane function and growth, using UGA codon for tryptophan, passing through “bacterial-retaining” filters, and, unlike typical bacteria, unable to be cultured on artificial media in the laboratory. In addition, many of the mycoplasmas pathogenic for humans and animals possess extraordinary specialized tip organelles that mediate their intimate interaction with eukaryotic cells (133).

The plant pathogenic phytoplasmas are known to be transmitted by more than 100 species of insects, including leafhoppers (a primary vector), planthoppers, and psyllids. They may also be seed-borne.

Diseases caused by phytoplasmas show symptoms of general yellowing, stunting, witches-broom growth of auxiliary buds, and change of flowers into leaves.

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# 11

## Interactions Between Host Plants and Fungal and Bacterial Pathogens

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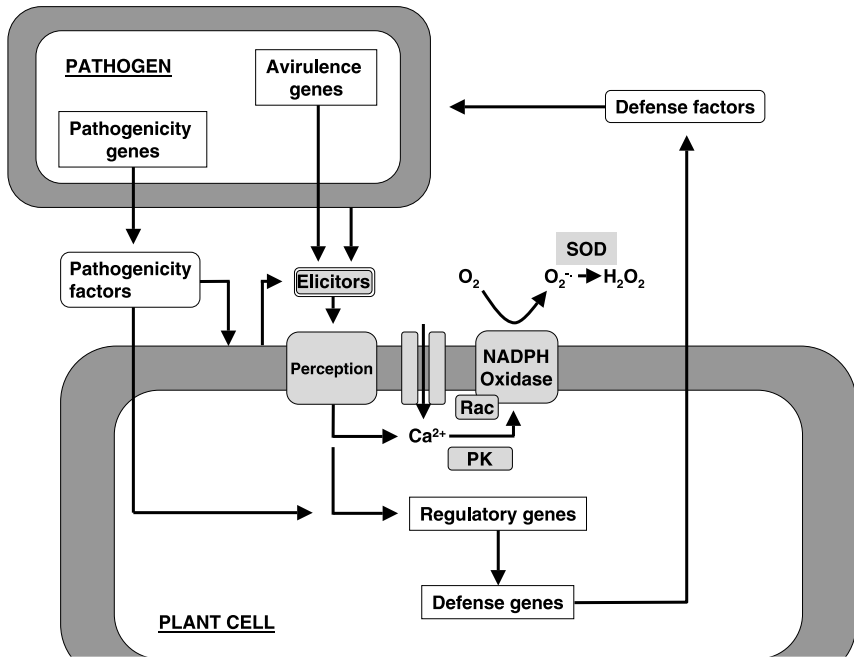
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### I. INTRODUCTION

Plants are, in general, resistant or immune to the majority of pathogens in the environment as they exhibit nonhost resistance: i.e., they are not an appropriate host for the pathogen that attacks them. Thus, resistance is the rule and susceptibility is the exception in biotic interactions. This resistance is based on a complex network of constitutive and inducible defense reactions. Only a certain number of host–pathogen pairs exist in which the pathogen has evolved the capability to resist or prevent a specific plant response (host compatibility). Defined plant cultivars have developed resistance against distinct pathogen races (cultivar resistance; see Refs. 1–3). In these well-studied host–pathogen interactions resistance is based on the presence of corresponding pathogen avirulence genes (*Avr* genes) and plant resistance genes (*R* genes) according to Flor's gene-for-gene hypothesis (4). During this process, defined products (ligands) of the *Avr* gene are secreted into the apoplast or the cytoplasm of the plant cell, where they interact with *R* gene products that often function as receptors (Fig. 1). These receptors are located intracellularly or on the plasma membrane.



**Figure 1** Plant–pathogen interactions. Among various plant responses, alterations in ion channels as well as the production of reactive oxygen species, the so-called oxidative burst, are depicted as early and central components of the plant defense response.

In general, the plant response to a pathogen is based on its ability to recognize signature molecules produced by the pathogen. These molecules are termed *elicitors* and, on one hand, originate from the pathogen (exogenous elicitors) and include peptides, glycoproteins, lipids, and oligosaccharides. In addition, endogenous elicitors are released from the plants' surface during pathogen attack. Race- and cultivar-specific elicitors are encoded by the avirulence gene of the pathogen (3). Most, but not all, proteins encoded by *R* genes recognize Avr proteins through leucine-rich repeat (LRR) domains and initiate signaling through kinase domains or interaction with kinases. A signal transduction pathway for bacterial flagellin elaborated in 2002 includes a LRR receptor kinase, as well as a mitogen-activated protein (MAP) kinase cascade that activates defined transcription factors (5).

Plants are equipped with both constitutive and inducible defense mechanisms. The former include constitutive properties such as the strength and thickness of cell walls and the presence of preformed antibiotics such

as polyphenols. On the other hand, induced resistance involves newly formed toxic chemicals and physical barriers. Research during the past years revealed that plant defense mechanisms in species and cultivar resistance are surprisingly similar. On pathogen recognition, regulatory genes initiate a multicomponent defense response whose elements are activated in a highly controlled temporal and spatial manner. Typical components of such responses include the production of phytoalexins, accumulation of (hydrolytic) pathogen-related (PR) proteins, induction of signal pathways, reinforcement of the cell wall, production of reactive oxygen species, and finally programmed cell death and systemic acquired resistance (SAR). In Fig. 1, the rapid activation of ion fluxes and an oxidative burst, the massive plant-derived production of reactive oxygen species, putatively by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, is depicted as a general result of the interaction between elicitors and receptors (6). In compatible interactions, these defense responses are not initiated or are activated only at a later stage, allowing the pathogen to exert its negative impact on growth and development of the plant. In this chapter we concentrate on signal pathways in response to bacteria and fungi as well as on systemic reactions after pathogen attack or inoculation with rhizosphere bacteria. We also discuss the effects of specific and nonspecific toxins in disease development.

## II. SIGNAL TRANSDUCTION

During the past several years, molecular, genetic, and biochemical studies have identified key players in the signaling pathways leading to plant defense responses. In this chapter, we focus on recent advances and discuss the central role of salicylic acid, jasmonic acid, and ethylene in plant resistance to pathogens.

### A. Phosphorylation

In many signal transduction pathways protein phosphorylation and/or dephosphorylation is known to play an important role. In vivo phosphorylation experiments and studies with pharmacological inhibitors have demonstrated that protein kinases and phosphatases are crucial for activation of early defense responses. A crucial role for protein phosphorylation has been suggested by the isolation from tomato of the *Pto* gene, which encodes a serine/threonine kinase, and from rice of the *Xa21* gene, which encodes a leucine-rich-repeat transmembrane receptor kinase (7). Plant defense responses are frequently triggered by a signaling network of



cross-talking pathways that may be interlinked at specific components. Current opinion is that the increase in the cytosolic  $\text{Ca}^{2+}$  concentration, which occurs within seconds after elicitation, is a master regulator required for many subsequent signaling steps. The oxidative burst, MAP kinase activation, defense gene activation, and phytoalexin production, whether alone or in combination, were shown to be inhibited in the presence of  $\text{Ca}^{2+}$ -chelating or  $\text{Ca}^{2+}$  channel-inhibiting compounds (8–10). Important milestones were tobacco plant expression of aequorin as a transgene, which showed an increase in cytosolic  $\text{Ca}^{2+}$  after nonspecific elicitation, and identification of  $\text{Ca}^{2+}$ -inward channels that responded to nonspecific and race-specific elicitors in parsley and tomato, respectively. However, the cellular machinery that transforms these changes in  $\text{Ca}^{2+}$  concentrations into gene expression is poorly characterized (11, 12).

Protein kinase C isoforms and calmodulin-dependent protein kinases have been characterized in detail as  $\text{Ca}^{2+}$  modulators in animals. In the context of innate immunity, protein kinase C activity is required for induction of the defense signal-related oxidative burst in macrophages (13). Little information is available about the function of protein kinase C in plants; it seems possible that calcium-dependent (but calmodulin-independent) protein kinases (CDPKs) fulfill its role.

Another important subset of kinases are CDPKs, which are a class of serine/threonine protein kinases that are unique to plants and some protists (14). The large CDPK gene family suggests that isoenzymes confer different specificities and function in multiple signaling pathways. The CDPKs are conserved in structure. They consist of a kinase catalytic domain, a conserved junction domain that functions as autoinhibitor, a calmodulin-like domain that in most cases contains conserved  $\text{Ca}^{2+}$  motifs, and an N-terminal variable domain. It should be noted that although CDPKs seem to be involved in response to several environmental stresses, and induction of CDPK messenger ribonucleic acid (mRNA) has been reported (14), there has been no previous evidence that CDPKs participate in activating plant defenses in plant–pathogen interactions.

In many eukaryotes, the transduction of defense-associated signals is induced by protein phosphorylation involving mitogen-activated protein kinases (MAPKs). Mitogen activated protein kinase and immediate upstream activators, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK), constitute a functionally interlinked MAPK cascade (8,9,15). Although many plant MAPK, MAPKK, and MAPKKK homologs have been identified on the basis of sequence conservation and functional complementation in yeast, their precise physiological functions in plants are mostly unknown. Elevation of MAPK activity has been detected in plant cells after exposure to various environmental stresses.

Recent reports have noted the activation of MAPK after race-specific and nonspecific elicitation. At least two phosphoproteins are implicated in the salicylic acid (SA) signaling pathway of tobacco. One of these proteins appears to work upstream of SA, whereas the other works downstream. An SA-induced protein kinase (SIPK) was shown to be activated at the enzyme level by various pathogen-associated stimuli, including two elicitors and a cell wall-derived (CWD) carbohydrate elicitor from *Phytophthora* spp., and bacterial harpin (16). In protein SIPK is activated in a gene-for-gene specific manner by the Avr9 peptide from *Cladosporium fulvum* in transgenic tobacco expressing the cognate resistance gene *Cf-9* and in tobacco mosaic virus- (TMV)-infected Xanthi nc carrying the cognate resistance gene *N* (17). Additionally, SIPK is activated to high levels by wounding. Thus, this kinase appears to be involved in multiple signaling pathways.

Some stimuli, such as the CWD elicitor, rapidly and transiently induce another MAPK, wounding-induced protein kinase (WIPK). Interestingly, the dramatic rise in WIPK activity after TMV infection or elicitor treatment is preceded by and requires both increases in WIPK transcription and translation, as well as posttranslational phosphorylation. By contrast, activation of SIPK, like that for MAP kinases in yeast and animals, is regulated strictly at the posttranslational level by dual phosphorylation of threonine and tyrosine residues (9). However, it remains to be determined whether these MAPK activation events are mediated through MAPK cascades consisting of specific MAPKs, MAPKKs, and MAPKKKs in plant cells.

## B. Salicylic Acid and Reactive Oxygen Species

The story of how salicylates, which include the pain relieving component salicylic acid (SA) present in willow tree bark, found their way from pharmacological studies to the laboratories of plant pathologists is circuitous. Plant scientists initially postulated that salicylic acid is an endogenous regulator of flowering. However, it is only in the last decade that convincing evidence was obtained for endogenous salicylic acid's having a regulatory role(s) in plant processes including thermogenesis and disease resistance (18, 19). Since then, a great deal of attention has been directed to elucidating salicylic acid's role in plant disease resistance.

The signaling pathways involved in the initiation and maintenance of the hypersensitive response (HR) and systemic acquired resistance (SAR) are still poorly understood. In the late 1970s and early 1980s, White and colleagues demonstrated that treatment of tobacco with SA or its derivative, aspirin, induced *PR* gene expression and enhanced resistance to pathogens such as tobacco mosaic virus (TMV). Strikingly, exogenously

supplied SA induced the same set of nine genes that are activated systemically TMV infection (20). In 1983 Van Loon first raised the possibility of a link between SA and SAR; he suggested that the accumulation of PR proteins is mediated in the plant by “an aromatic compound that mimics the action of SA” (21). However, in the early 1990s, it became apparent that SA is the endogenous compound that operates in the signaling pathway for plant defense. After TMV infection, SA accumulates to high levels at the site of infection, with a subsequent, but much smaller rise in level in the uninfected systemic tissues. In tobacco, this increase paralleled the transcriptional activation of *PR* genes in both inoculated and uninoculated leaves. An increase in SA levels in the phloem of tobacco necrosis virus- or *Colletotrichum lagenarium*-infected cucumber plants was also shown to precede the development of SAR (22, 23). Further confirmation of SA’s being an SAR signal was found in studies on NahG plants (24).

### 1. Is Salicylic Acid the Mobile Signal?

It has been known for some time that the signal for establishment of systemic acquired resistance (SAR) is transported from the pathogen-inoculated leaf to uninoculated leaves via the phloem. Reports of SA accumulation that occurred in parallel to or even preceded *PR* gene activation and SAR development in uninfected leaves of TMV-inoculated tobacco, combined with the detection of SA in the phloem of pathogen-infected tobacco or cucumber, suggested that SA may be the systemic signal for SAR (22). However, although these experiments clearly demonstrated a correlation between SA and SAR, they do not prove that SA is the long-distance mobile signal. Currently, strong evidence that SA may be the long-distance SAR signal has risen from an elegant experiment in which the translocation of labeled SA was monitored in TMV-infected tobacco. Since the final step in SA biosynthesis in tobacco is the O<sub>2</sub>-dependent hydroxylation of benzoic acid by benzoic acid 2-hydroxylase, Shulaev and coworkers were able to label the SA synthesized in TMV-inoculated lower leaves by enclosing them in an <sup>18</sup>O<sub>2</sub>-rich environment. Subsequent analysis of the upper uninoculated leaves indicated that almost 70% of the SA was <sup>18</sup>O-labeled and therefore had been synthesized in and transported from the TMV-inoculated leaf (24).

Furthermore, the biosynthesis and transport of SA have been studied by administering <sup>14</sup>C benzoic acid to cucumber cotyledons infected with *Colletotrichum lagenarium*. In these experiments <sup>14</sup>C-labeled SA was detected in upper uninoculated leaves before the development of SAR. In 1997, it was shown that methyl salicylate, produced from SA on TMV

infection of tobacco, may function as an airborne signal. Alternatively, it may be translocated through the vascular system. After methyl salicylate's conversion back to SA, it activates defense responses in uninfected tissues and possibly even neighboring plants (25).

Despite the strong correlations from these studies on salicylate biosynthesis and transport, they do not rule out the possibility that salicylates are simply translocated in parallel with a yet to be identified signal molecule. This possibility is supported by the observation that the signal for SAR development moved out of *Pseudomonas syringae*-infected cucumber leaves *before* any increase in SA level could readily be detected in the phloem sap (26). Grafting experiments between NahG and wild-type tobacco have also suggested that SA is not the long-distance signal. When a NahG rootstock (which is unable to accumulate SA) was inoculated with TMV, the uninoculated systemic leaves of the wild-type scion still showed SAR (27). However, these results need to be interpreted with caution. Although it has been shown that catechol, produced from SA by salicylate hydroxylase, cannot substitute for SA, it is not clear whether the residual SA in NahG plants may be able to act as a long-distance messenger.

Studies of transgenic tobacco expressing the cholera toxin gene, a known modulator of signaling pathways dependent on hetero-trimeric G-proteins, also suggest that SA is not the translocated SAR signal. These plants constitutively accumulate high levels of SA, express *PR* genes, show enhanced resistance, and develop spontaneous lesions. However, SAR was not observed when a wild-type scion was grafted onto a transgenic rootstock, even though the rootstock accumulated high levels of SA (28). Thus, further studies, such as the isolation of SA biosynthetic genes and the identification of mutations targeting the pathways for SA metabolism and transport, are required to clarify whether SA functions as a long-distance signal.

## 2. Mechanisms of Action

Whether or not SA emerges as the mobile SAR signal, it does appear to be required for the establishment and maintenance of SAR. This conclusion is based on results from the NahG grafting experiments described previously. Systemic acquired resistance did not develop in a NahG scion after infection of the wild-type rootstock with TMV. However, the mechanism by which SA induces SAR is still unclear. Previous studies have demonstrated that SA binds and inhibits tobacco catalase activity both *in vitro* and *in vivo*. Thus, one possible function of SA is to inhibit the hydrogen peroxide- ( $H_2O_2$ )-degrading activity of catalase, thereby leading to an increase in the endogenous level of  $H_2O_2$ , which is generated by

photorespiration, photosynthesis, oxidative phosphorylation, and the HR-associated oxidative burst (29).  $\text{H}_2\text{O}_2$ , or other reactive oxygen species (ROS) derived from it, could then serve as second messengers to activate the expression of plant defense-related genes, such as *PR-1*. This hypothesis is currently the subject of intense debate as outlined later.

### 3. Reactive Oxygen Species and Plant Defense

In plants,  $\text{H}_2\text{O}_2$ , superoxide radicals ( $\text{O}_2^{\cdot-}$ ), and hydroxyl radicals ( $\text{OH}\cdot$ ) are thought to play key roles in defense responses (6, 30, 31). After infection, plants resistant to the invading pathogen develop a sustained increase of ROS level. In a manner analogous to their participation in macrophage or neutrophil action, these ROS may be involved in directly killing invading pathogens. In addition, increases in  $\text{H}_2\text{O}_2$  level have been shown to induce the crosslinking of cell wall proteins and to enhance the peroxidase-catalyzed synthesis of lignin, thereby creating a physical barrier against pathogens (32, 33).

Reactive oxygen species can also serve as second messengers for the activation of defense gene expression. For example, elevated ROS levels induce the genes for glutathione-S-transferase, glutathione peroxidase, and polyubiquitin, as well as peroxidases, catalases, and other enzymes involved in ROS scavenging, chilling tolerance, and pathogen resistance (34). Currently, the mechanism(s) by which redox signaling activates these genes is a matter of debate. The ability of ROS and thus the cellular redox state, to activate plant defenses may parallel the mechanism by which oxidative stress induces the genes associated with animal immune and inflammatory responses. Activity of at least two transcription factors, NF- $\kappa$ B and AP-1, have been shown to be regulated by the cellular redox state; whether these proteins are activated directly by  $\text{H}_2\text{O}_2$  or indirectly by thiol metabolites, such as glutathione, is not clear. To date, the only genes shown to be directly regulated by ROS are those in the bacterial *oxyR* and *sox* regulons (35).

Another line of early plant defense responses that may be triggered by ROS is the induction of cell death. Treatment of soybean suspension cells with high concentrations of  $\text{H}_2\text{O}_2$  (6–10 mM) was shown to cause cell death, an effect that may be enhanced by the addition of SA or the catalase inhibitor 3-aminotriazole. In contrast, another study has suggested that  $\text{O}_2^{\cdot-}$ , but not  $\text{H}_2\text{O}_2$ , is crucial for the induction of cell death. To determine the mechanism by which spontaneous lesions develop on the leaves of the *Arabidopsis* lesion simulating disease (*lsd1*) mutant in the absence of pathogen infection, these plants were treated with  $\text{O}_2^{\cdot-}$  or  $\text{H}_2\text{O}_2$  generating or scavenging systems. Strikingly, elevated levels of  $\text{O}_2^{\cdot-}$ , but not of

H<sub>2</sub>O<sub>2</sub>, were able to induce lesion formation (36). Despite repeated suggestions that ROS are involved in the signaling pathways leading to apoptosis and/or programmed cell death in animals, there still is no conclusive evidence that ROS are required for the execution of cell death. Indeed, it has recently been suggested that ROS may be associated with, but not directly responsible for, apoptosis in animal cells.

#### 4. H<sub>2</sub>O<sub>2</sub> and Salicylic Acid: What is the Source and What is the Signal?

It has been hypothesized that SA binds to catalase, inhibits its activity, and thereby increases the intracellular concentration of H<sub>2</sub>O<sub>2</sub>, which might then serve as a second messenger for the induction of a defense response. In contrast, recent reports have suggested that *PR* gene induction during the HR and SAR may not be activated by SA-mediated increases in H<sub>2</sub>O<sub>2</sub> concentration. At the site of infection, SA levels can reach 150 μM, a concentration sufficient to cause substantial inhibition of catalase and of ascorbate peroxidase, the other major H<sub>2</sub>O<sub>2</sub>-scavenging enzyme (37). However, no decrease in catalase activity could be detected in pathogen-inoculated leaves. By contrast, the concentration of SA in uninfected systemic tissue is probably too low to increase H<sub>2</sub>O<sub>2</sub> levels through the inhibition of catalase or ascorbate peroxidase, unless SA is concentrated in a subcellular compartment.

Recent studies using transgenic tobacco plants have also suggested that the SA-mediated inhibition of catalase and increased level of H<sub>2</sub>O<sub>2</sub> are not involved in the activation of defense responses. When catalase expression was suppressed in leaves of transgenic plants through sense cosuppression or antisense suppression, most plants did not exhibit constitutive *PR* gene expression. Additionally, H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>-inducing chemicals were unable to induce *PR* expression in NahG plants although they could activate *PR-I* genes in wild-type tobacco. On the basis of these results, SA appears to act downstream of H<sub>2</sub>O<sub>2</sub>, rather than the reverse. Furthermore, it was demonstrated in the late 1990s that high levels of H<sub>2</sub>O<sub>2</sub>, as well as ozone or ultra violet (UV) light treatment, stimulate SA biosynthesis. Thus, H<sub>2</sub>O<sub>2</sub> may play a role in the activation of *PR* genes by increasing SA levels (38, 39).

It is believed that all organisms utilize signaling cascades to transduce oxidative stress and that they frequently respond to stress by strengthening their antioxidative systems. Whether SA plays a role in either process in plants is unknown. It has been proposed that alterations in the cellular redox state, due to changes in the glutathione or plastoquinone pools or the levels of metabolites such as nicotinamide or SA, serve as redox messengers

in plants. On the other hand, phenol-based anti-inflammatory drugs (including SA and aspirin) are thought to act, at least in part, as antioxidative compounds and direct ROS scavengers (40). Thus, it is possible that SA functions, in part, by acting as an antioxidant. In this capacity, SA could help contain the oxidative damage associated with lesion formation and/or spread during the HR. This action would closely resemble SA's antioxidative role in inflamed mammalian tissues. It is noteworthy that in NahG plants, lesions that develop on TMV infection are larger than in wild-type plants. However, an antioxidative role of SA *in planta* remains to be proved.

If the predominant mechanism by which SA induces defense responses is not through increased H<sub>2</sub>O<sub>2</sub> levels caused by interaction with Fe-containing proteins involved in redox control, how is the SA signal perceived and transmitted? Despite all efforts there is currently no convincing model that explains SA's mode of action. Currently, a growing body of evidence suggests that SA plays a pleiotropic role in general redox regulation and/or signal transduction (41).

### C. Jasmonic Acid, Ethylene, and Systemin

Jasmonates, i.e., jasmonic acid (JA), its methyl ester, certain amino acid conjugates, glucose esters, and hydroxylated forms, occur ubiquitously in all plant species. Jasmonates are known to promote diverse processes including fruit ripening, senescence, tuber formation, tendril coiling, and pollen formation. Most importantly, jasmonates constitute a major signal in stress-induced gene expression (42, 43). Herbivore attack, damage by mechanical wounding, desiccation, or pathogen attack triggers the elevation of level of endogenous JA, which in turn induces the expression of specific jasmonate-responsive genes to fight insects and, in some cases, necrotrophic fungi. The role of jasmonates in plant defense responses has been best characterized with respect to the wound-induced expression of proteinase inhibitor (*pin*) genes, which inhibit the digestive serine proteinases of herbivorous insects (44, 45). The JAs also induce vegetative storage proteins, a thionin (*Thi2.1*), and plant defensins (PDFs). Octadecanoid-deficient mutants of *Arabidopsis* sp. (e.g., *opr3*) and tomato (*Lycopersicon esculentum*) are significantly more susceptible to wounding or herbivore damage than corresponding wild-type plants. Furthermore, JA induces biosynthesis of many volatile compounds, mainly terpenoids, that may contribute to plant-plant communication and/or plant-fungi or plant-insect interactions (46, 47).

Biosynthesis of JA has been characterized in detail. The most important key enzyme in JA production is allene oxide synthase (AOS).



The *AOS* gene has been cloned from many plants including *Arabidopsis* sp. Allene oxide synthase is the first enzyme in the branch pathway leading to the biosynthesis of JA and catalyzes the production of unstable allene epoxides that cyclize to form cyclopentenone acids, the precursors for JA (48, 49). Wounding, PDA, and JA induce the expression of both *Arabidopsis* sp. and flax *AOS*. Lipoxygenase-derived hydroperoxides of linolenic and linoleic acids are substrates in this reaction. From 13S-hydroperoxy-9(Z),11(E),15(Z)-octa-decatrienoic acid (HPOT) derived from  $\alpha$ -linolenic acid as a substrate, the jasmonate precursor 12-oxophytodienoic acid (OPDA) is synthesized in the reaction initiated by AOS. Recently OPDA has been recognized as an early but independent center of biological activity within the octadecanoids.

The molecular mechanisms that follow the increase of endogenous JA level but precede gene expression, are not known as yet. Several transcription factors respond to JA, but a direct interaction remains to be proved. Nevertheless, the JA signaling pathway represents an excellent example for cross-talk within plant signaling systems. Salicylic acid, an elicitor of pathogenesis-related gene expression, induces *Arabidopsis* sp. *AOS* but represses flax *AOS*, indicating the existence of diverse signaling pathways among plant species with regard to jasmonate-mediated signal transduction. In 2002, SA was suggested to be a negative regulator of proteinase inhibitor genes, thus providing “cross-talk” between the signaling systems involved in pathogen defense and predator defense (50).

*Arabidopsis* sp. *AOS* is also induced by the phytohormone ethylene. It is believed that ethylene interacts with jasmonates to regulate proteinase inhibitor genes in the wound response of tomato (51, 52). Noteworthy, not only JA, but also ethylene are formed when plants are wounded or attacked by pests or pathogens. Several mutants defective in either the production or the perception of ethylene or JA have increased susceptibility to several pests and pathogens. Thus, both signal pathways are required for pathogen resistance. A possible mechanism for JA- and ethylene-dependent defenses is through their synergistic interaction in the induction of many defense-related genes, including *PR5*, *PDF1.2*, and basic chitinase (*CHI-B*), and of a heveinlike protein. It should be noted, however, that whereas JA and ethylene cooperate synergistically in the activation of wound-related defense responses, some JA responses are antagonized by ethylene (53).

Jasmonate action and/or biosynthesis is also closely linked to the 18-amino acid polypeptide hormone systemin, which has been shown to play an essential role in systemically signaling the expression of defense genes in tomato plants in response to herbivore attack and other mechanical wounding (54). The polypeptide is derived from a 200-amino acid precursor, prosystemin, through proteolytic cleavage. The crucial role of



systemin in the signaling pathway was shown by transforming tomato plants with a prosystemin antisense gene that is regulated by the cauliflower mosaic virus 35S promoter. The transgenic plants produced high levels of prosystemin antisense mRNA, resulting in a severely compromised wound response. *Manduca sexta* larvae readily consumed the transgenic plants, whereas wild-type plants exhibited a normal defense response by inhibiting larval growth. On the other hand, plants transformed with a cauliflower mosaic virus 35S–prosystemin gene, with the prosystemin mRNA in the sense orientation, produced high levels of prosystemin mRNA in plants (55). These transgenic plants exhibited constitutive expression of >20 systemic wound response proteins in leaves and seemed to be in a permanently wounded (and protected) state.

Together with JA, systemin appears to be a key signal that activates leaf cells for defense against predators. It induces a signaling cascade that produces intracellular alterations in ion transport, intracellular increases in the activities of MAP kinases and phospholipase A<sub>2</sub>, induction of calmodulin, and increases in intracellular Ca<sup>2+</sup>. Current opinion is that these early events result in the release from membranes of linolenic acid, its conversion to jasmonic acid by way of the octadecanoid pathway, and the activation of defense genes (45, 56).

An important step in the initiation of these early events is the interaction of systemin with a putative receptor. A systemin binding site has been reported to be present in isolated membranes of a wild tomato species, *Lycopersicon peruvianum* (45). However, unlike for most mammalian hormones, the molecular action of systemin is still poorly described and needs to be resolved.

### III. HYPERSENSITIVE RESPONSE AND LOCAL ACQUIRED RESISTANCE

Signal transduction and cell death mechanisms are generally well conserved in evolution and can thus be identified throughout the living world from unicellular to mammalian organisms (57, 58). The response of plants to avirulent pathogens and nonpathogens has been termed *hypersensitiveness* by Stakman (1915; see Ref. 59) to describe rapid cell death of leaf tissue at and around the invasion site. Later, the term *hypersensitive response* (HR), which comprises rapid, localized death of plant cells in association with the functional suppression of pathogen growth by the accumulation of defense-related metabolites and proteins at the infection site (60), was introduced. It is now generally accepted that this general defense strategy

of plants is a form of genetically defined, programmed cell death (*pcd*; see Refs. 61 and 62) with analogies to animal cell death, and that it is hypersensitive only with respect to the highly efficient perception and guard mechanisms that constantly survey the environment for potential aggressors.

Although the HR is a common feature of many resistance reactions, it is not an obligatory component. Several reactions such as those mediated by the *mlo* gene in barley and *Cf* genes of tomato under high humidity precede the induction of HR symptoms or occur without visible HR, respectively (63). The HR lesions are also formed spontaneously in the absence of pathogens in various mutants (62), a finding that indicates that the scores is under preexisting genetic control. In addition to the well-studied HR, *pcd* is observed during symptom formation of the virulent infections (64) and is involved in various developmental processes such as deletion of suspensor and aleurone cells, formation of leaf lobes and perforations, and cell death in xylem tracheary elements (61, 65).

Programmed cell death has to be differentiated from necrotic cell death. Necrosis is accidental cell death activated by overwhelming physical or chemical trauma. It is disorganized and uncontrolled and usually results in the death of large groups of cells or whole tissues. Necrotic cells lose membrane and organelle integrity early, and deoxyribonucleic acid (DNA) fragmentation, if it occurs, is random and appears late. The key characteristics of *pcd* are the cell autonomous decision to die and active participation of the cell in its own execution. Apoptosis in animal cells is a form of *pcd* involving a series of well-organized events requiring active cell participation. It is the basis for normal tissue remodeling as well as the end result of toxic insults. The key features of apoptosis are nuclear shrinkage, DNA fragmentation, late loss of organelles, cell shrinkage, and breakup of the cell and nucleus into membrane-bound apoptotic bodies, which are taken up by the surrounding cells (66). At the molecular level, the fragmentation of nuclear DNA into approximately 50 kb pieces (oligonucleosomes) is involved in this cell disassembly. Several genes and proteins including *fas*, the *bcl* family, caspases, and other proteases, and the release of cytochrome *c* from mitochondria into the cytosol have been identified as regulators of *pcd* in different species (67).

Findings in recent years point to similarities in the processes involved in plant and animal *pcd*. However, as a result of the physical restriction of the cell wall, plant cells do not fulfill all criteria defining apoptosis in animal cells. Thus, the more general term *programmed cell death* is often favored in plants instead of *apoptosis*. Caspase-like protease activity is involved in the *N*-gene-mediated HR in tobacco, in the ozone-induced cell death in *Arabidopsis thaliana*, and in cell death in barley cultures (68, 69).



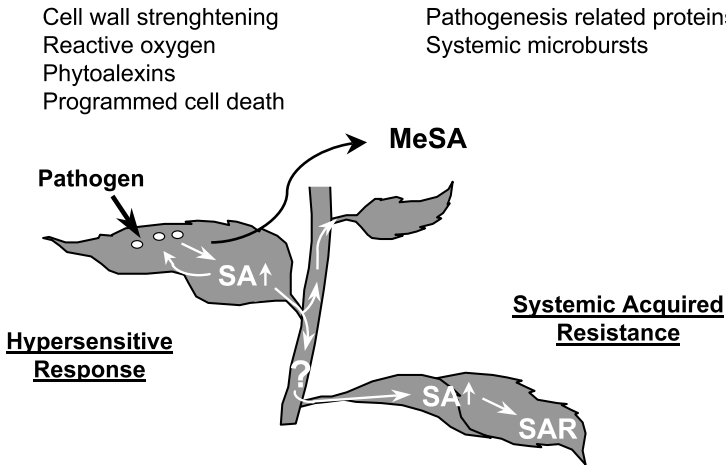
that activating signals are present in the border cells between dead and healthy tissue. A burst of ethylene biosynthesis (1-aminocyclopropane-1-carboxylic acid [ACC] content, ethylene emission) is stimulated in parallel in certain plant–pathogen interactions (74), catalase-deficient tobacco (75), and ozone-sensitive plants (69). It usually precedes the accumulation of other signals such as jasmonate and salicylate. A second, persistent burst of ROS accumulation (phase II) is only found when ethylene is present above a certain (low) threshold level. Ethylene may act either by activating Nox activity or by blocking enzymes or low-molecular-weight compounds inhibiting Nox activity (Fig. 2). Activation could occur by phosphorylation in the stimulation of Nox activity, as known for mammalian cells (76). On the other hand, inactivation of Nox activity could be mediated by ethylene inhibition of the accumulation of polyamines that are known Nox inhibitors in animal tissues (77). It will be one of the most intriguing questions for the next few years to analyze the regulation of Nox and other potential ROS-forming enzymes, e.g., cell wall peroxidases, by ethylene, jasmonate, and other signals.

Models first proposed in 1998 predict that plants can resist oxidative stress as long as the ethylene and salicylic acid pathways are not activated in excess or when they are sufficiently inhibited by jasmonate (78–80). In addition, both ethylene and salicylate pathways have to be active to avoid cell death to propagate (Fig. 2). In the absence of the oxidative stress, the balance between the signaling pathways aims to inhibit activation of the cell death–promoting effect of ethylene and salicylate. It seems that suppressing parts of the salicylate pathway by ethylene in nonoxidative conditions could protect plants from spontaneous cell death under optimal growth conditions. During oxidative stress, the balance between the signaling pathways is altered toward ethylene and salicylate to allow cell death to occur. It seems that the biosynthetic kinetics of both ethylene and SA is critical for the regulation of the signaling balance and the concomitant cell death.

#### **IV. SYSTEMIC ACQUIRED RESISTANCE AND INDUCED SYSTEMIC RESISTANCE**

##### **A. Systemic Acquired Resistance**

Associated with the hypersensitive response (HR) and a common response to necrogenic pathogen infection is the development of systemic resistance to subsequent pathogen attack. This induced resistance, or systemic acquired resistance (SAR), results in broad-spectrum, long-lasting immunity in noninfected tissue that provides protection against not only the inducing



**Figure 3** Defense responses to pathogen infection. Pathogen infection of resistant plants generally results in the formation of necrotic lesions and restricted pathogen growth and spread, the hypersensitive response (HR). A variety of defense responses are induced locally around the sites of infection. An oxidative burst precedes formation of necrotic lesions. Additional defense responses in surrounding cells include the induction of genes for pathogenesis-related (PR) proteins, peroxidases, and enzymes involved in cell wall strengthening and biosynthesis of phytoalexins. Some of these genes are also activated systemically and are believed to play a role in the development of systemic acquired resistance (SAR). The synthesis and accumulation of salicylic acid (SA) appear to be necessary for the activation of several of these defense responses, both locally and systemically. It is still unclear whether SA is a long-distance mobile signal in SAR. Most recently, methyl salicylate (MeSA), which is synthesized from and metabolized to SA, was shown to act as an airborne signal that activates defense mechanisms in distal leaves and possibly even neighboring plants. However, at room temperature, MeSA is a liquid and could be translocated through the vascular system of the plant, just as SA.

pathogen but a spectrum of pathogens including viruses, bacteria, and fungi (Fig. 3). This mechanism contrasts with the so-called gene-for-gene resistance, which is described as a pathogen-specific interaction in which a specific plant resistance gene confers resistance to a particular pathogen strain (41). Despite considerable efforts, several decades after discovery of SAR the signal transduction events that lead to its establishment are not well defined. However, certain advances at determining the molecular basis for SAR have been made (81). The onset of SAR is in most cases, i.e., plant systems, correlated with the coordinate induction of genes termed *SAR genes*. Several families of pathogenesis-related (PR) proteins are

dramatically induced during SAR. Some of these proteins are hydrolytic enzymes (e.g.,  $\beta$ -1,3-glucanases [PR-2] and chitinases [PR-3]), the functions of other PR proteins have yet to be determined. However, most of the PR proteins have been shown to possess antimicrobial activity in vitro or the ability to enhance disease resistance when overexpressed in transgenic plants (82). In tobacco and *Arabidopsis* sp. PR-1a and acidic PR-1 are the most abundant and tightly correlated SAR markers, respectively. The accumulation of SA (see previous discussion) that accompanies SAR has been shown to be essential for SAR signaling. In tobacco, application of exogenous SA (and aspirin) induces *PR* gene expression and partial resistance to pathogens such as tobacco mosaic virus (TMV) (83). In addition, increased levels of endogenous SA correlate with the development of resistance and the induction of *PR* genes in cucumber and tobacco. Salicylic acid also induces the same set of genes that are activated systemically by TMV infection (81).

The importance of SA in plant defense responses was further documented by using transgenic tobacco and *Arabidopsis* sp. expressing the *nahG* gene, which encodes the SA-metabolizing enzyme salicylate hydroxylase from *Pseudomonas putida*. These plants accumulate little, if any, SA and as a consequence show reduced or no *PR* gene expression, do not establish SAR, and are compromised in their ability to prevent pathogen growth and spread from the primary infection site. In addition, suppression of gene-for-gene resistance in NahG plants has led to the suggestion that common SA-dependent signaling events may function in both SAR and gene-for-gene resistance mechanisms (83). The key role of SA in the activation of resistance was further underscored by the demonstration that *Arabidopsis* sp. plants become susceptible to avirulent fungal pathogens when phenylalanine ammonia lyase (PAL) enzyme activity is specifically inhibited. Since PAL performs the first step in SA biosynthesis and resistance can be restored in these PAL-inhibited plants by treatment with exogenous SA, increased susceptibility is presumably due to a block in SA synthesis (84).

In addition to defense reactions already apparent on induction of resistance, other responses are manifested only after challenge inoculation. Potentiation is expressed as a faster and greater activation of defense-related genes after infection of induced plants with a challenging pathogen. For instance, tobacco plants exhibiting pathogen-induced SAR show enhanced expression of *PR-10* and *Pal* genes on challenge with a pathogen. Ethylene, SA, and jasmonate have been shown to act as potentiating signals of defense-related gene expression. Salicylic acid was reported to be a potentiator of pathogen-induced defense responses such as *Pal* gene expression and phytoalexin accumulation that do not respond directly to SA (85).

In contrast to resistance-inducing derivatives of SA, biologically inactive analogs of SA failed to potentiate elicitor-induced *Pal* gene expression in cultured parsley cells, indicating that the resistance inducers may act in part by augmenting the activation of certain defense-related genes. Jasmonate has been shown to potentiate the elicitor-induced accumulation of active oxygen species in parsley cells. In rice, jasmonate has been demonstrated to potentiate the induction of *PR-1* gene expression and resistance against the fungal pathogen *Magnaporthe grisea* by low doses of the resistance-inducing synthetic compound 2,6-dichloroisonicotinic acid (INA). Ethylene acts as a potentiator of SA- and pathogen-induced *PR-1* gene expression in *Arabidopsis* sp. Moreover, in regard to the expression of the *Pin* gene in tomato and the *PR-5* gene in tobacco, ethylene has been reported to have a potentiating effect on the action of jasmonate, and vice versa. In *Arabidopsis* sp., a marked synergy between ethylene and jasmonate was demonstrated for the induction of certain defense genes. An overview on priming effects is given by Ref. 86.

Pathogen-associated cell death may be a necessary requirement for biological SAR activation. However, the induction of SAR by the exogenous application of activators such as SA or INA does not involve cell death, thereby suggesting that these compounds stimulate the SAR pathway downstream from cell death. Biological induction of SAR requires infection by a pathogen that causes (programmed) cell death. The correlation between rapid hypersensitive cell death and plant disease resistance has been widely described (87). The HR is associated with a massive increase in the generation of reactive oxygen species (oxidative burst), which both precedes and accompanies lesion-associated host cell death. It has been proposed that systemic "microbursts" may lead to activation of SAR (88). However, the connection between cell death and the establishment of SAR is unclear, although SA-dependent processes appear to play an important role. For example, *NahG Arabidopsis* sp. and tobacco plants, which cannot accumulate SA, exhibit spreading lesions after pathogen infection. That result supports a role for SA or SA-dependent processes in the manifestation of pathogen-associated cell death (89).

Many genetic approaches have been launched to understand regulation of SAR. In addition to environmental stress, such as exposure to UV light and ozone, the inappropriate expression or repression of endogenous or foreign genes in plants can lead to the constitutive expression of defense genes, the activation of SAR, and, in several cases, the spontaneous development of HR-like lesions. In most of these cases, the constitutive SAR and spontaneous lesion phenotypes are associated with elevated levels of endogenous SA (90, 91). However, none of these transgenes is anticipated to directly participate in SA biosynthesis. Rather, their expression may induce



metabolic stress, which in turn elevates SA levels, resulting in constitutive SAR. Alternatively, it has been suggested that, in some cases, expression of these transgenes mimics part of the defense signaling pathway, which then activates SA biosynthesis.

Several *Arabidopsis* sp. mutants that exhibit SAR and contain constitutively high levels of SA have been identified. Interestingly, the lesion simulating disease (*lsd*) and accelerated cell death (*acd2*) mutants also spontaneously develop HR-like lesions, whereas the constitutive expression of *PR* genes (*cpr1*), constitutive expression of *PR* (*cep1*), and constitutive immunity (*cim3*) mutants do not. Since the constitutive SAR phenotype of these mutants is suppressed by the presence of the *nahG* gene, elevated levels of SA appear to have a causal role in the development of constitutive SAR (90, 91).

In contrast to these constitutive SAR mutants are the SAR-compromised mutants, of which only the allelic *npr1* class appears to affect the SA signal transduction pathway. Mutations in this gene prevent both the development of SAR and the induction of *PR* genes by SA. The recessive nature of these mutations suggests that the wild-type protein acts as a positive regulator of the SA signal transduction pathway. Recently, *NPR1* was shown to encode a unique 60-kd soluble protein containing ankyrin repeats, which facilitate protein–protein interactions. Since one of the *npr1* mutations is in these repeats, Npr1 likely functions in signal transduction by interacting with other proteins. The *npr1* mutants are also insensitive to the plant activators 2,6-dichloroisonicotinic acid and benzothiadiazole, a finding that supports the biochemical evidence that all three compounds induce plant defense responses via the same signal transduction pathway (92).

Whereas SA appears to be required for SAR, analysis of the preceding mutants, as well as transgenic plants, has suggested that SA per se is not required for HR development. The *lsd2* and *lsd4* mutants are capable of developing HR in the presence of the *nahG* gene. Moreover, TMV infection leads to HR in NahG tobacco plants. These lesions, however, are larger and more diffuse than those exhibited by wild-type plants. Furthermore, these lesions eventually spread to the stems, suggesting that the NahG plants have lost their ability to limit the HR. Hence, although SA may not play a causal role in lesion development, it may participate in restricting lesion spread. In sum, screening of signal transduction mutants has identified several key components of SAR and HR signaling events and complements biochemical and physiological approaches (93).

Recent advances in the understanding of events that lead to SAR have led to the development of novel SAR-activating compounds as well



as the generation of genetically modified plants with heightened resistance to disease. The use of chemical activators of SAR as well as the identification and analysis of SAR mutants have greatly contributed to our understanding of induced resistance. Application of INA to tobacco induces the same spectrum of resistance and *PR* genes as is seen after SA treatment or TMV infection. Since INA does not stimulate SA biosynthesis and is able to induce SAR in NahG transgenic tobacco plants, it likely mimics SA or functions at a step downstream of SA. Other studies have suggested that INA is a functional analog of SA (94). Very recently, a new synthetic crop protection agent, a benzothiadiazole (BTH), which is highly effective at inducing enhanced disease resistance, has been developed. Benzothiadiazole elicits the same set of defense responses induced by SA. Thus, BTH and INA both appear to be functional analogs of SA (95). The induction of SAR by the exogenous application of activators such as SA or INA does not involve cell death, thereby suggesting that these compounds stimulate the SAR pathway downstream from cell death.

## B. Induced Systemic Resistance

Induction of systemic resistance by pathogens is well known, colonization of the rhizosphere by selected nonpathogenic *Pseudomonas* sp. bacteria can trigger a systemic resistance as well. This specific form of induced resistance is referred to as *rhizobacteria-mediated induced systemic resistance* (ISR). In *Arabidopsis* sp., *Pseudomonas fluorescens*-mediated ISR has been shown to be effective against different types of pathogens, including the bacterial leaf pathogen *Pseudomonas syringae* pv. tomato (Pst), the fungal root pathogen *Fusarium oxysporum* f. sp. *Raphani*, and the oomycetous leaf pathogen *Peronospora parasitica*. Other ISR-promoting bacteria are *Burkholderia cepacia*, which is frequently used as a biocontrol agent. *Pseudomonas* (WCS417r)-mediated ISR seems to be associated with potentiation of phytoalexin accumulation, resulting in higher phytoalexin levels after infection by the fungal pathogen *F. oxysporum* f. 539 sp. *dianthi*.

Little is known about ISR-associated defense genes (96). The molecular mechanisms underlying rhizobacteria-mediated ISR in *Arabidopsis* sp. are to a large extent unknown. Induced systemic resistance signal transduction follows an SA-independent pathway that confers resistance in the absence of *PR* gene expression (97). In contrast to SAR, ISR requires responsiveness to both jasmonates and ethylene (see previous discussion). However, like SAR, rhizobacteria-mediated ISR is dependent on the *NPR1* gene, indicating that NPR1 not only is required for the expression of

*PR* genes that are activated during SAR (in a SA-dependent manner), but also functions in the jasmonate- and ethylene-dependent activation implicated in rhizobacteria-mediated ISR (96). Surprisingly, SA-, ethylene-, or jasmonate-responsive defense-related gene transcripts are not induced during ISR. On the other hand, that observation does not automatically mean that no SA-, ethylene-, or jasmonate-dependent defense responses are elicited. The resistance against *Pst* induced by very low concentrations of SA, ACC, or MeJA may result either from the accumulation of other defensive compounds before challenge inoculation and/or from potentiation of defense-related gene expression after challenge with a pathogen (priming). These mechanisms could be the same as the ones controlling WCS417r-mediated ISR (96, 98). In sum, the nature of the defense responses contributing to the rhizobacteria-induced resistance is unclear and its elucidation requires further work.

## V. HOST-SPECIFIC AND HOST-NONSPECIFIC PHYTOTOXINS

A great number of plant pathogenic fungi and bacteria are known to produce phytotoxins that contribute to disease symptom development in infected plants. Some phytotoxins such as victorin or HC-toxin are essential for pathogenicity; others such as tabtoxin only contribute to virulence of the toxin-producing pathogen but are not essential for pathogenicity. In general, phytotoxins are divided into two groups: the host-specific toxins that affect only the host plants of the toxin-producing pathogen and the unspecific toxins that also cause symptoms on other plants. In this chapter some examples of both groups are introduced.

### A. Host-Specific Toxins

The first host-specific toxins (HSTs) were identified more than 60 years ago. They are produced by a number of fungal pathogens, mainly *Alternaria* and *Cochliobolus* species. In most cases they are determinants of pathogenicity: i.e., races of the pathogen lacking toxin production are not virulent to the host plant. As mentioned earlier host-specific toxins cause symptoms only in the host plant of the toxin-producing pathogen and do not affect other plants or nonsusceptible cultivars of the host plant. Host-specific toxins vary in structure from low-molecular-weight molecules to high-molecular-weight metabolites such as proteins. In principle, host

sensitivity is determined by one single gene and is based on two different mechanisms:

1. The susceptible host expresses a receptor for the toxin. This receptor is missing in nonsensitive plants.
2. The susceptible host lacks a detoxification mechanism that is present in nonsensitive plants.

## 1. T-Toxin

T-toxin is produced by *Cochliobolus heterostrophus* (= *Helminthosporium maydis* = *Bipolaris maydis*) race T, the causal agent of Southern corn leaf blight. The host plant maize is carrying Texas cytoplasm for male sterility (cms-T-maize). Figure 4 shows the polyketide structure of T-toxin. Interestingly, a structurally related phytotoxin (PM toxin) is produced by *Mycosphaerella zeae-maydis* (*Phyllosticta maydis*) and resembles T-toxin in its mode of action. In susceptible maize plants, T-toxin acts by affecting mitochondrial function. After T-toxin treatment oxidative phosphorylation is uncoupled, state 4 respiration is stimulated, and small molecules leak from the mitochondrion (99).

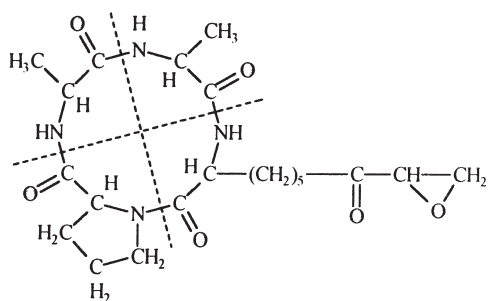


**Figure 4** T-toxin.

Analysis of the genome of cms-T mitochondria led to the discovery of the T-urf 13 gene responsible for T-toxin sensitivity. This gene is absent in mitochondria of maize other than cms-T. The *T-urf* 13 gene codes for a 13-kd protein (URF 13) that is located in the mitochondrial membrane in tetrameric form. Binding of T-toxin to the URF 13 protein results in the formation of pores, eventually resulting in the loss of membrane semipermeability (100).

## 2. HC-Toxin

HC-toxin is a cyclic tetrapeptide (Fig. 5) produced by *Cochliobolus carbonum* (= *Helminthosporium carbonum* = *Bipolaris zeicola*), which causes Northern leaf spot and ear rot of maize. Of the three races of the pathogen only race 1 produces HC-toxin. Although HC-toxin is a virulence factor, it is not a pathogenicity factor since the non-toxin-producing races of *H. carbonum* also cause symptoms on the host plant. However, lesions are smaller compared to infection with race 1 (101). Insensitivity to the



**Figure 5** HC-toxin.

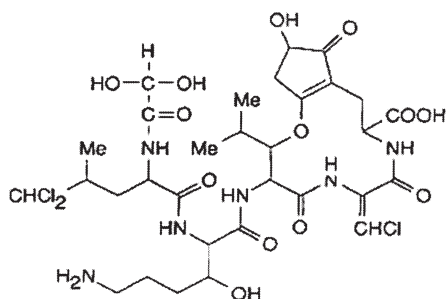
toxin is determined by the *Hm1* gene coding for HC-toxin reductase, an enzyme inactivating HC-toxin by reducing the carbonyl function (102).

HC-toxin inhibits histone-deacetylase (103), leading to the accumulation of hyperacetylated core histones. The way in which the inhibition of this enzyme affects gene expression is not yet clear, but there is evidence that HC-toxin exerts its toxic activity by repression of resistance mechanisms. This repression may be due to an inhibited expression of resistance genes (100).

### 3. Victorin

Victorin (=HV-toxin) is a toxin produced by *Cochliobolus victoriae* (= *Helminthosporium victoriae*), a fungal pathogen that causes victoria blight of oats. There exist several forms of victorin that are very similar, and all are complex chlorinated cyclic pentapeptides. The structure of victorin C, which is the major phytotoxin produced by *C. victoriae*, is given in Fig. 6. The most compelling evidence for victorin's being a pathogenicity factor is that isolates of the fungus that do not produce the toxin are nonpathogenic to oat. The sensitivity of the host plant is conditioned by the dominant allele at the *Vb* locus. Recessive genotypes are not sensitive to the toxin and are therefore resistant to *C. victoriae*. Thus, victoria blight occurs only if the fungus produces victorin and the host carries a dominant allele at the *Vb* locus (100).

Victoria blight only affects oat plants that carry the *Pc2*-resistance gene against the crown rust fungus *Puccinia coronata*. The disease appeared in 1945 after the introduction of the oat variety Victoria, which contained this gene for resistance to *P. coronata*. Resistance to *P. coronata* and sensitivity to victorin could not be separated, and therefore it was concluded that the *Pc2* gene and the *Vb* gene are either identical or at least tightly linked



**Figure 6** Victorin.

(100). In the host plant victorin induces several reactions that resemble defense reactions, e.g., deposition of callus, formation of the phytohormone ethylene, and phytoalexin synthesis (see Refs. in 100). Therefore, the question arises why victorin acts as toxin and not as avirulence factor. The answer may be that victorin induces a variation of programmed cell death (PCD) that is also characteristic for the hypersensitive reaction induced by specific elicitors. This PCD is characterized by shrinkage of cells, DNA laddering, and activation of proteases and is a genetically controlled and highly organized form of cellular suicide. However, in contrast to the hypersensitive reaction, the victorin-induced cell death does not result in pathogen defense but induces the destruction of host tissue. Victorin produces all the external symptoms as well as the histochemical and biochemical changes in the host induced by the pathogen, as there are structural changes of the cell wall structure, electrolyte loss of the cells, increased respiration, and decreased growth and protein synthesis (104).

The main target of victorin action seems to be the mitochondria of the host plant. In 1995 it was demonstrated that victorin binds to components of the mitochondrial glycine decarboxylase complex (105). This binding to glycine decarboxylase may contribute to victorin-induced dysfunction of the plant mitochondria.

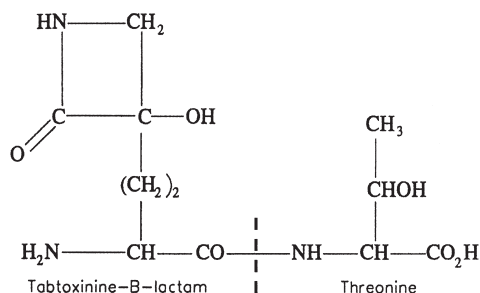
## B. Unspecific Toxins

In contrast to host-specific toxins the unspecific toxins are toxic not only to the host of the toxin-producing pathogen but also to other plants. The mechanisms of action are manifold and are described for selected examples in the following section.

## 1. Effect on Host Enzymes

Several unspecific toxins exert their toxicity via the inhibition or activation of host enzymes.

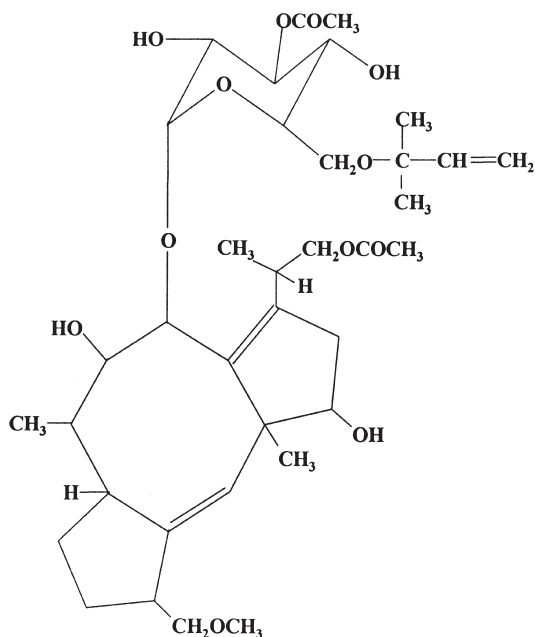
**Tabtoxin.** Tabtoxin is a monocyclic  $\beta$ -lactam produced by *Pseudomonas syringae* pv. tabaci, coronafaciens, and garcae (Fig. 7). It is associated with the symptoms of wildfire disease on tobacco and halo blight disease on oat. The toxic component of tabtoxin is tabtoxinin- $\beta$ -lactam, which is released from tabtoxin by microbial or plant aminopeptidases. For tabtoxin it was shown that its toxic subunit, tabtoxinin- $\beta$ -lactam, inhibits glutamine synthetase (106), thereby increasing the concentration of ammonia in the chloroplast where it is generated via nitrite reductase. Ammonia is known to uncouple photophosphorylation, thereby accelerating photosynthetic electron transport. Reduction of the oxidized nicotinamide adenine dinucleotide phosphate ( $\text{NADP}^+$ ) pool results in electron transfer from photosystem I to molecular oxygen, triggering the formation of ROS. This effect of tabtoxin is amplified by the inhibition of ribulose-1,5-bisphosphate-carboxylase by the toxin, which leads to a decrease in NADPH oxidation via the Calvin cycle.



**Figure 7** Tabtoxin.

**Fusicoccin.** Fusicoccin is a nonselective phytotoxin produced by *Fusicoccum amygdali*, the cause of the twig blight disease of almond and peach trees. It is a tricyclic glycosidic diterpenoid (Fig. 8) that was first isolated by Ballio and coworkers (107). Fusicoccin causes leaf wilt during *Fusicoccum* sp. infection because it induces stomata opening, thereby leading to uncontrolled transpiration (108). Interestingly the fusicoccin effects resemble the effects of auxins. Nevertheless, apparently their receptors are not identical.

The primary target of fusicoccin action is the  $\text{H}^+$  adenine triphosphatase ( $\text{H}^+$ -ATPase) located in the plasmalemma. The activation of this



**Figure 8** Fusicoccin.

enzyme by fusicoccin was reported by several authors for many different plants (109–111). Fusicoccin has been a helpful tool to elucidate regulative mechanisms of the  $\text{H}^+$ -ATPase. The C-terminal region of the  $\text{H}^+$ -ATPases functions as an autoinhibitor of enzyme activity and is sensitive to several effectors that change the conformation of this domain, thus eliminating the inhibition. Results obtained by Lanfermeijer and Prins (112) point to a modulation of the C-terminal autoinhibitory domain by fusicoccin, suggesting that fusicoccin-induced stimulation of the  $\text{H}^+$ -ATPase is due to the displacement of the C-terminal region.

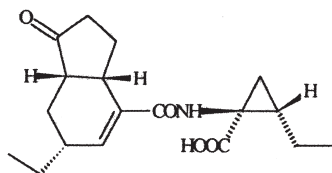
In the last few years many efforts have been made to identify the fusicoccin receptor in plants. Noteworthy, a 30-kd membrane-associated protein was identified. Sequencing studies with the 30-kd protein revealed an amino acid sequence with high homology to a corn 14-3-3 protein (113–115). A common theme of 14-3-3 proteins is the regulation of protein kinases. Together with the fact that  $\text{H}^+$ -ATPases can be phosphorylated by  $\text{Ca}^{2+}$ -dependent kinases these observations imply a role for protein kinases in the fusicoccin-induced signal transduction pathway. Olivari and associates (116) demonstrated in radish seedlings that binding of fusicoccin

to its binding protein induces an interaction between the  $H^+$ -ATPase and the fusicoccin-receptor complex that seems to be necessary for ATPase activation. Taken together these results point to a short fusicoccin signaling pathway in which the fusicoccin binding protein belonging to the class of 14-3-3 proteins binds to the plasma membrane  $H^+$ -ATPase, inducing its activation only in the presence of fusicoccin. Protein kinases may be involved in this activation process by changing the phosphorylation status of the C-terminal autoinhibitory domain of the  $H^+$ -ATPase, which is the target for the binding of the 14-3-3 protein.

## 2. Phytohormones

**Coronatine.** Coronatine is a polyketide toxin (Fig. 9) produced by several pathovars of *Pseudomonas syringae*, e.g., pv. glycinea, tomato, and maculicola. It induces chlorosis on a wide variety of plant species. Other symptoms induced by coronatine are induction of hypertrophy, inhibition of root elongation, and stimulation of ethylene production (117). In tomato coronatine induces the accumulation of proteinase inhibitors in the vacuole (117). Coronatine consists of two distinct components: the polyketide coronafacic acid and coronamic acid, an amino acid derived from isoleucine with a cyclopropane moiety. Coronatine reveals structural homology to methyl jasmonate, a plant hormone derived from the octadecanoid pathway. Several research groups showed that in various plant species coronatine induces biological responses analogous to that of methyl jasmonate (118). For this reason coronatine is believed to function as a molecular mimic of methyl jasmonate.

Genes for biosynthesis of coronatine are located on a 90-kb plasmid in *P. syringae* pv. glycinea. Regulation of biosynthesis is interesting because maximal production of the toxin occurs at 18°C, consistently with symptom development in the field (119). It could be shown that temperature regulates coronatine biosynthesis at the transcriptional level. This regulation works through a modified two-component regulatory system in which the *CorR* and *CorP* gene products function as response regulators and the *CorS* gene codes for a histidine protein kinase with homology to sensor proteins (120).

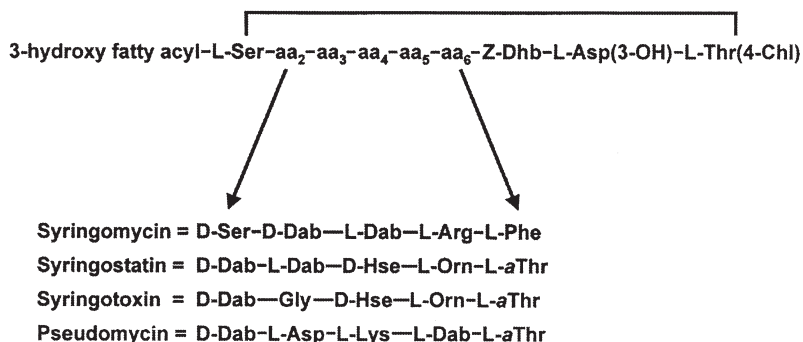


**Figure 9** Coronatine.



### 3. Destruction of Membranes via Pore Formation

**Syringomycin.** Syringomycin is a cyclic lipodepsinonapeptide (see Fig. 10) produced by *Pseudomonas syringae* pv. *syringae*. It consists of a polar peptide head and a hydrophobic fatty acid tail. There exist three forms of syringomycin, depending on the length of the fatty acid. Interestingly, syringomycin has a chlorinated amino acid (4-chloro-threonine) that is important for its toxic activity (121). Related toxins produced by *Pseudomonas syringae* pv. *syringae* are syringotoxin, syringostatin, and pseudomycin (see Fig. 10). Syringomycin induces necrosis on host tissue where the primary target is the plasma membrane. Because of its amphiphilic character syringomycin is inserted into the membrane, leading to pore formation. The syringomycin-induced pores are freely permeable for cations, measurable as an influx of  $H^+$  and  $Ca^{2+}$  and an efflux of  $K^+$  at nanomolar concentrations of the toxin (117). Biophysical investigations (122, 123) revealed that at least six molecules of syringomycin form one ion channel. The influx of  $Ca^{2+}$  leads to the activation of several signaling cascades in the plant cell (see Sec. V.B.1.).



**Figure 10** Syringomycin and syringopeptin.

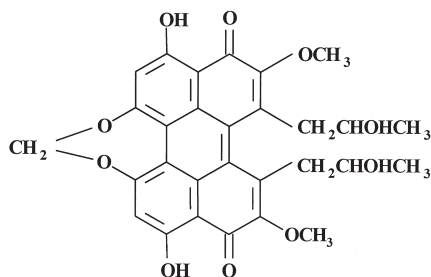
The benefit for the bacteria lies in the toxin-induced efflux of nutrients from the cytoplasm to the intercellular space. Yet another advantage for the toxin-producing pathogen is that because of the biosurfactant activity of syringomycin the spread of the bacteria on the plant surface is facilitated because the surface tension of water is reduced and nutrients are concentrated at solvent interfaces (124). Syringomycin production is regulated by the nutritive situation and/or plant signal molecules. Specific phenolic glycosides of the host plant (e.g., arbutin in pear) induce toxin synthesis, an effect that is stimulated by several sugars (117).

*Syringopeptin.* Syringopeptin is a lipodepsipeptide also produced by *Pseudomonas syringae* pv. *syringae*. In contrast to syringomycin it contains 22 or 25 amino acids, depending on the toxin-producing bacterial strain. Phytotoxic activity of syringopeptin is highly comparable to that of syringomycin and is mainly based on pore formation in the plant plasma membrane (117). Syringopeptin as well as syringomycin are secreted by the bacteria via an ABC transporter protein encoded by the *syrD* gene (125). This transport is driven by the hydrolysis of ATP at the cytoplasmic domain of the ABC transporter.

#### 4. Oxygen Activation by Phytotoxins

Several phytotoxins exert their toxicity by the activation of oxygen, leading to the production of reactive oxygen species (ROS, see Chap. 2) in the plant cell (126). Reactive oxygen species, like the superoxide radical, the hydroxyl radical, hydrogen peroxide, and singlet oxygen, are toxic to cells and react rapidly with most organic molecules, such as fatty acids, proteins, and nucleic acids. When ROS are produced within the cells the detoxification system may be overloaded and oxidative processes take place. The peroxidative breakdown of unsaturated fatty acids is induced by hydroperoxy radicals derived from superoxide at the Gouy-Chapman layer of lipophilic membranes as well as by OH radicals or electron donor- $\text{H}_2\text{O}_2$  complexes ("crypto-OH") and also by singlet oxygen, which directly produces lipid hydroperoxides (127). For a review of the formation and activity of ROS see Ref. 128.

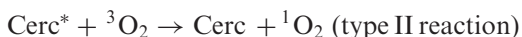
*Cercosporin.* Cercosporin is a perylenequinone toxin (Fig. 11) produced by several phytopathogenic *Cercospora* species. These fungi cause severe plant diseases worldwide, for example, *Cercospora* leaf spot of sugar beet (*C. beticola*), purple seed stain of soybean (*C. kikuchii*), and frog-eye leafspot of tobacco (*C. nicotianae*). Cercosporin plays an important



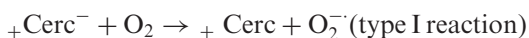
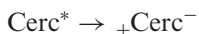
**Figure 11** Cercosporin.

role in pathogenicity of *Cercospora* species (129). Mutants of the fungus that are deficient in cercosporin biosynthesis cause only weak symptoms on host plants as compared to those of wild-type isolates. Symptoms induced by cercosporin treatment are consistent with the symptoms caused by *Cercospora* infection.

So far, all evidence indicates that cercosporin exerts its toxic effect on plant cells through the peroxidation of membrane lipids, finally resulting in membrane destruction and cell death (130, 131). Toxicity of cercosporin is strictly light-dependent: killing of plant cells by the toxin occurred only in the light, whereas even high concentrations of cercosporin did not induce cell death in the dark. This light dependency of cercosporin action is due to its photosensitizing activity (132). Cercosporin is activated by visible wavelengths of light and generates reactive oxygen species (singlet oxygen, superoxide), which are toxic to living cells, in photodynamic reactions of both type I and type II:

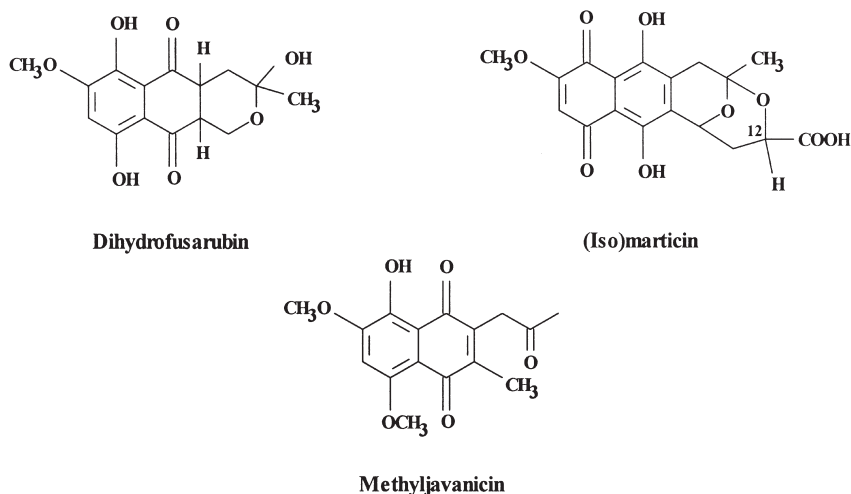


or:



There are several reports that indicate that after light activation of cercosporin singlet oxygen is the predominant toxic agent because quenchers of singlet oxygen such as diazabicyclooctane (DABCO) delayed cercosporin-induced killing of cells (131). Several other phytopathogenic fungi were shown to produce photosensitizing toxins. For example, photoactive perylenequinones have been isolated from several *Cladosporium* species, as well as from *Alternaria* species (133). In 2003 rubellin D, an anthraquinone metabolite isolated from the barley pathogen *Ramularia collo-cygni*, was shown to induce the formation of reactive oxygen species in a light-dependent reaction (134).

**Naphthazarines.** The naphthazarin toxins are naphthoquinone metabolites produced by *Fusarium solani*. Examples are marticin, isomarticin, javanicin, and dihydrofusarubin, shown in Fig. 12. *Fusarium solani* is a common soil-borne fungus that causes root rot symptoms in peas, beans, lentils, and cucurbits (135, 136). Kern (137) and Parisot and colleagues (138) reviewed the effects of *F. solani* naphthazarin toxins on plant systems. They reported that such toxins are readily extracted from injured tissue of



**Figure 12** Some naphthazarines.

infected pea plants in quantities sufficient to damage healthy tissue. Disease symptoms became visible after treatment of healthy pea seedlings with marticin and isomarticin. Both toxins can be transported in the plant, causing leaf necrosis.

The naphthazarin toxins are also known to enhance membrane permeability; that effect may be the main reason for the accumulation of stress-related compounds in xylem fluid of blight-diseased citrus (139). In addition to chlorophyll degradation the toxin affected chloroplast membranes by causing granal stack disorganization and swelling of inter-granal membranes. Concomitantly an increase in plastoglobuli became visible. In the case of dihydrofusarubin the formation of ROS via reductive processes at photosystem I, resembling the action of the herbicide methylviologen, was shown. Albrecht and coworkers (140) clearly demonstrated that this naphthazarin toxin interacted with the photosynthetic electron transport chain of spinach chloroplasts, thereby leading to the formation of ROS. Furthermore, they proved that superoxide was formed in a light-dependent reaction on photosystem I comparable to the reaction of methylviologen. As a consequence, NADP<sup>+</sup> reduction of spinach chloroplasts was inhibited, whereas ATP formation was accelerated in the presence of dihydrofusarubin. Besides reduction at photosystem I, naphthazarines can also be reduced in light-independent reactions via enzymatic reactions (diaphorases), reduction by cytochrome P450, and dithiols (126).

### C. Autoresistance of Toxin-Producing Pathogens

Looking at unspecific toxins the question arises why toxin-producing pathogenic fungi and bacteria are not damaged by their own toxins. In many cases this property is not well understood, but several efforts have been made to define the mechanisms of autoresistance. Some examples are described in the following paragraph.

#### 1. Cercosporin

*Cercospora* species accumulate high concentrations (up to millimolar) of cercosporin but are not killed by this toxin in the light. Daub and associates (141) demonstrated that hyphae of *Cercospora* species actively reduced the toxin to a nontoxic form. Cercosporin excreted by the fungus spontaneously reoxidizes into the photoactive toxic form. Additionally vitamin B<sub>6</sub> seems to play a role in resistance of *Cercospora* sp. to singlet oxygen (129, 142).

#### 2. Syringopeptin and Syringomycin

Resistance of *Pseudomonas syringae* pv. *syringae* to lipodepsipeptide toxins may be explained by the effective export system in the bacterial membrane. As mentioned (E.2.c.2.) syringopeptin and syringomycin are secreted by the bacteria via an ABC transporter protein encoded by the *syrD* gene. In actinomycetes and fungi the export of antibiotics by these ABC transporters often is the basis for autoresistance (143, 144).

#### 3. Tabtoxin

Tabtoxin irreversibly inhibits glutamine synthetase (see earlier discussion). Resistance of the *Pseudomonas* species that produce tabtoxin has been associated with a modification of the enzyme. Glutamine synthetase in toxin-producing strains of the pathogen is adenylated and therefore less susceptible to the toxin (145).

Another possibility is that the bacteria achieve resistance to tabtoxin through the expression of  $\beta$ -lactamases (146). These enzymes cleave the  $\beta$ -lactam moiety of the toxin, rendering it nontoxic.

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# 12

## Allelopathy

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### I. INTRODUCTION

The term *allelopathy* is derived from the Greek (*allellos*, of each other; *pathos*, to suffer) to indicate the injurious effect of one on another. Molisch (1) coined the term in 1937 and his definition referred to both the detrimental and beneficial biochemical interactions among all classes of plants, including microorganisms. In 1996 the International Allelopathy Society defined *allelopathy* as follows: “*Any process involving secondary metabolites produced by plants, microorganisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects*” (2).

The taxonomist DeCandolle (3) investigated the chemical interactions between different species of higher plants. He observed that root exudates of several plants had harmful effects on neighboring plants and therefore influenced the composition of plant communities. He noticed in corn fields the negative influence of thistles on oat plants as well as the growth inhibition of flax due to *Euphorbia* and *Scabiosa* sp. He also carried out experiments on several allelopathic phenomena.

The science of allelopathy progressed in the 20th century, especially as a result of the work of Muller and his coworkers (summarized in Refs. 4 and 5). Chemicals released from plants that exert allelopathic influences are termed *allelochemicals* or *allelochemics*. Most of them are secondary metabolites. According to Rice (5) the chemical compounds identified as allelopathic agents can be divided into the following groups: simple water-soluble organic acids, straight chain alcohols, aliphatic aldehydes, and



ketones; simple unsaturated lactones; long-chain fatty acids and polyacetylenes; naphthoquinones, anthraquinones, and complex quinones; simple phenols, benzoic acid, and derivatives; cinnamic acid and derivatives; coumarins; flavonoids; tannins; terpenoids and steroids; amino acids and polypeptides; alkaloids and cyanohydrins; sulfides and mustard oil glycosides; purines and nucleosides.

Some allelochemicals such as phenolic acids also have structural functions (e.g., as intermediates of lignification) or play a role in the general defense against plant pathogens and herbivores. The functions of natural terpenoids in the interrelationships between organisms are summarized by Harrewijn and coworkers (6). Allelopathic compounds leave plants by volatilization, exudation from roots, leaching by rain, or decomposition of residues. Another possible plant-plant movement of allelochemicals is through root grafts, fungal bridges, or haustoria of parasitic vascular plants (5).

A clear proof of an allelopathic relationship requires, according to Rice (5), the fulfilment of the following conditions: (a) The synthesis of an allelopathic compound takes place in the donor plant; (b) the release of these compounds in an allelopathic active or inactive form to the environment follows as second step; microorganisms can convert inactive compounds into active allelochemicals; (c) the next step is the distribution and possibly accumulation in the environment; (d) an acceptor plant incorporates the compounds; (e) the result is an allelopathic reaction. Other types of interferences such as the competition for light, water, or nutrients, e.g., the inhibition of flax (*Linum usitatissimum*) by *Camelina sativa*, a crucifer, are not allelopathic. This effect is due to competition for nutrients.

The direct mode of action of allelochemicals, which includes effects on various aspects of plant growth and metabolism, has received fairly wide attention (7). Important sites and processes are known to be attacked or influenced by allelochemicals. These include structural aspects such as membrane integrity and permeability; cytological and ultrastructural as well as biochemical changes, e.g., protein synthesis, leghaemoglobin synthesis, and nitrogen fixation; specific enzyme activity; and pigment synthesis. In addition physiological events such as germination of pollen and spores, phytohormones and their balance, respiration, water relations of plants, mineral uptake, photosynthesis, and stomatal movements can be changed. Abenavoli and colleagues (8) assume that allelochemicals such as coumarin inhibit the cell cycle and/or promote senescence.

No generally accepted well-defined methods exist for the verification of allelopathic activity (9). Because of the complexity of allelopathic interactions the validity of protocols based on a plant physiological approach

has been questioned. Some aspects of this problem are listed in the following paragraph (cf. Ref. 9).

Tests with isolated allelochemicals are difficult to evaluate. On one hand there may be an influence of the solvent due to the extraction method; on the other hand, often combined effects of several compounds exist. Several laboratory experiments indicate that mixture solutions of allelochemicals have greater effect than the same concentrations of the compounds applied separately.

The actual release rate of allelochemicals from living plants is difficult to estimate. They can be released continuously, can be released within specific periods, or can be triggered by external factors. The loss of allelochemicals due to degradation by microorganisms, plant uptake, or adsorption by soil particles is also unclear.

Biotic and abiotic factors can both influence the production of allelochemicals by the donor and modify the allelopathic effect on the receiver plant. Factors such as light, nutrient availability, water availability, pesticide treatment, and disease affect the amount of allelochemical produced by a plant. In general one can state that allelopathy and stresses interact under natural conditions.

Consequently, a holistic approach in which the experimental design is adapted to the species and the ecosystem under investigation has been recommended in recent years.

## II. EXAMPLES OF DIFFERENT TYPES OF ALLELOPATHY

In the following chapter several classical examples of allelopathy are introduced. A detailed description is found in Harborne (10). The following case studies represent the different types of distribution of allelochemicals: (a) distribution as volatiles, (b) diffusion from donor plant to roots of acceptor plants, and (c) leaching from leaves, e.g., by rain, and accumulation in the soil around the donor plant.

### A. Californian Chaparral

The Californian chaparral provides a famous example for allelopathy. This plant community is found along the coastal strip of Southern California and is adapted to a climate of hot, dry summers and relatively cold winters. Mainly evergreen shrubs are found in this locally restricted community because they are able to use a short vegetation period in spring and autumn

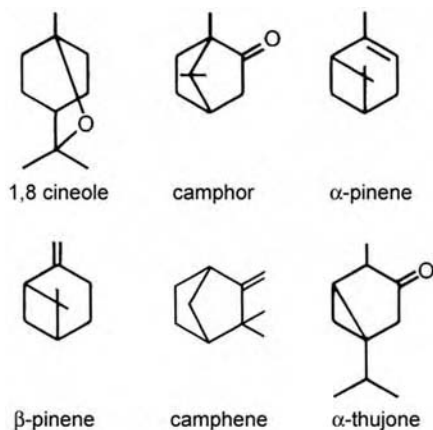


**Figure 1** Inhibitory zone (A–B) between *Salvia leucophylla* (left) and grassland (C). Between B and C the growth of grass is inhibited. (From Ref. 11.)

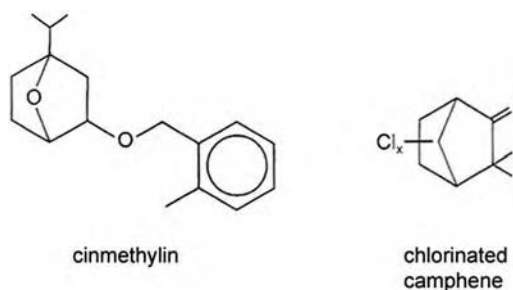
after the first rainfall. The Chapparal is adjacent to areas of natural, uncultivated grassland. The zonation of herbs around a thicket of shrubs is one of the most striking phenomena of this area. Figure 1 illustrates this phenomenon (11).

The first type of chapparal (coastal sage scrub) is dominated by shrubs that produce volatile terpenes such as *Salvia leucophylla* and *Artemisia californica*. The most effective compounds are 1,8-cineole and camphor, which inhibit plant growth and seed germination. Other volatiles include  $\alpha$ - and  $\beta$ -pinene and camphene (*Salvia* sp.) and *Artemisia* sp. ketone,  $\alpha$ -thujone, and isothujone (Fig. 2). These volatile terpenes are produced in large amounts in epidermal glands on the leaf and other parts. After release during heat they surround the plants as a vapor cloud. They can dissolve through wax and cutin layers on the surface of other plants and enter the cells. This capacity leads to a strong inhibition of germination, especially in annular grasses, which occur in the neighboring grassland area. The terpenes become enriched in dry soil. Degradation occurs only after rain, when soil organisms become active.

The toxicity of monoterpenes such as  $\beta$ -pinene results from interaction with lipid domains of membranes. Not only changes of membrane properties are observed; inhibition of the electron transport at photosystem II, destruction of the chloroplast envelope, as well as changes in the fat metabolism leading to changes in the galactolipid composition of chloroplast



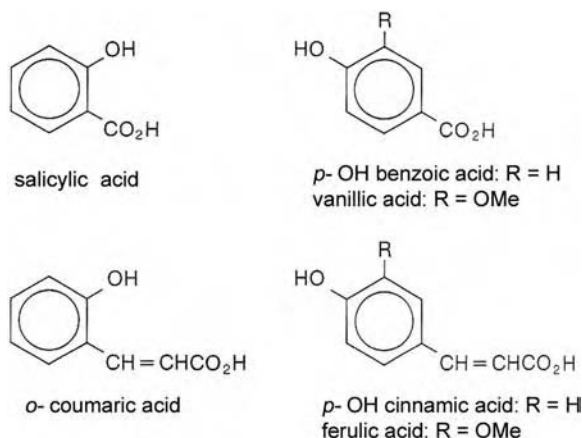
**Figure 2** Cyclic monoterpenes that have an allelopathic effect.



**Figure 3** Cinmethylin, a synthetic cineole herbicide, and chlorinated camphene (Toxaphen).

membranes are also observed. The phytotoxicity of *Artemisia* sp. terpenoids forms the basis of the development of several pesticides, among them cinmethylin (Fig. 3), an analog of 1,8-cineole. The highly active growth inhibitor blocks entry into mitosis. Chlorinated camphene was used first as an insecticide (commercial product Toxaphen or Camphechlor), then for 10 yr in legume cultures such as soy beans for the suppression of *Cassia obtusifolia*, before it was withdrawn. This product is a mixture of more than 177 polychlorinated C<sub>10</sub> compounds with Cl<sub>6</sub>- to Cl<sub>10</sub>-components.

The second type of chaparral (chamise chaparral) is dominated by hard-leaved evergreen shrubs, among them the chamise *Adenostoma fasciculatum* (Rosaceae) and *Arctostaphylos glandulosa* (Ericaceae). In their close vicinity no grasses grow, not even after strong rainfall. The reason for this are water-soluble phenolic compounds, which are washed out from



**Figure 4** Allelopathic water-soluble phenolic compounds.

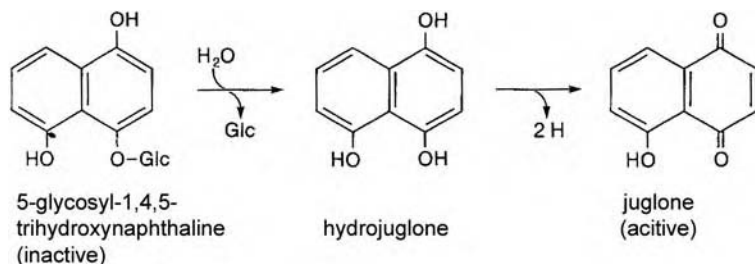
leaves and enriched in the soil. The most effective compounds are *p*-hydroxybenzoic acids and *p*-hydroxycinnamic acids, which inhibit seed germination of grasses and herbs (Fig. 4). In addition, phenol derivatives such as salicylic acid and *o*-coumaric acid play a role. The toxicity of phenolic acids is due to interference with auxin metabolism as well as changes of the membrane potential.

A striking ecological phenomenon in the Californian chaparral are cyclical changes of the vegetation caused by periodic natural fires, which occur in intervals of 10–40 yr and usually result from lightning or ignition. After the destruction of the shrub layer many annual species follow in the next vegetation periods because gemination inhibitors have been destroyed by heat.

However, most shrubs slowly regenerate from the surviving root buds and eventually produce seeds. Continuous growth of the shrubs, usually 5–6 yr after the fire, leads to the accumulation of toxins, which prevents seed germination, and the chaparral remains free of herbs until the next fire. This example illustrates the principle of autointoxication in established plant societies. This means that in several species germination of its own seed is inhibited and competition within the same species is prevented within its own habitat.

## B. Juglone of the Walnut Tree

Gaius Plinius Secundus, also known as Pliny the Elder (c.e. 23–79), already knew about the growth inhibition of quite a few plants in the direct vicinity



**Figure 5** Release of active juglone from the bound form.

of walnut trees (*Juglans regia*). These observations could not be explained before the 20th century. Most of them refer to the North American black walnut (*J. nigra*), but the effects are also seen in the European species, *J. regia*. Because the toxic effects are located within the area of root growth, it was first assumed that root exudations were responsible for the death of plants (e.g., cereals, potato, tomato, pine, but not raspberry or blackberry). Later work indicated that the toxic effects were actually due to leaching from the walnut leaves, stems, branches, and nut hulls of a bound toxin (the 4-glucoside of 1,4,5-trihydroxynaphthalene). This compound undergoes hydrolysis and oxidation in the soil by microorganisms, followed by the release of the active toxin, the naphthoquinone juglone (Fig. 5), which then kills any annual species growing in its vicinity. This means that the toxin occurs within the plant in a safe, nontoxic form, where autooxidation is prevented. It is only after leaching from the leaves and stems into the soil that it becomes active and can exert its effect.

The occurrence of juglone, a heavy staining water-soluble yellow pigment, is strictly limited to the green parts of the tree and is lost in dead tissue and ripe nuts. It is an important inhibitor of seed germination: a juglone solution at a concentration of 0.002% completely prevents germination of lettuce seed. Many plants (e.g., tomato, alfalfa) are killed if juglone is injected via the petiole.

The following experiment clearly demonstrates the toxicity of juglone on tomato seedlings as well as the importance of the presence of soil microorganisms to convert the precursor to the bioactive toxin: nut hulls of *J. regia* were ground and mixed with the substrate in which 2-wk-old tomato seedlings were planted. Half of the pots contained heat-sterilized soil; the other half, the untreated soil. Control plants were cultivated in the two different soils without nut hulls. After 2 wks only the plants in unsterilized soil mixed with nut hulls showed significant growth depression (Fig. 6). The assumption that any growth-depressing pathogen was present in the

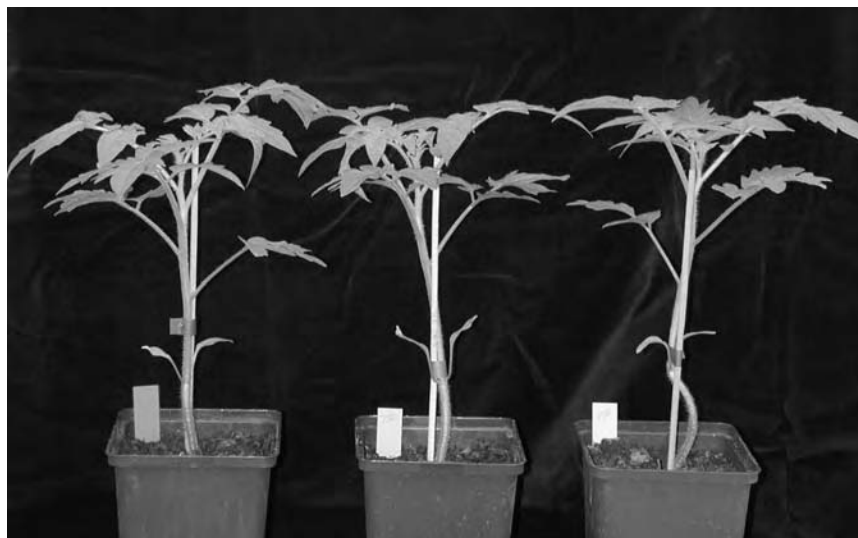


**Figure 6** Growth depression of tomato seedling in unsterilized soil mixed with nut hulls of *Juglans regia* (left) compared to soil–nut hull mixture with sterilized soil (right).

unsterilized soil can be excluded because control plants in soil without nut hulls grew normally (Fig. 7).

In the United States failures in reforestation were often linked to the allelopathic effects of *J. nigra*. Black walnut is grown there because it yields timber and because it grows fast, often together with  $N_2$ -fixing plants such as *Elaeagnus umbellata* and *Alnus glutinosa*. Alder already shows growth disturbances at a concentration of  $1\ \mu\text{mol/l}$  juglone: in a mixed stand after 8–13 yr death of alder plants could be observed (12). Juglone also affects the symbiotic partner of *A. glutinosa*, *Frankia* sp., which is responsible for nodulation and  $N_2$ -fixation. Depending on the isolate growth, nodulation,  $N_2$ -fixation, and root respiration are delayed.

Under natural conditions the effective concentration of juglone is dependent on the absorption rate by the soil and further microbial destruction. Schmidt (13) isolated from the soil close to a walnut tree a



**Figure 7** Tomato seedlings in a mixture of sterilized soil and nut hulls of *Juglans regia* (left), in sterilized soil without nut hulls (middle), and in unsterilized soil without nut hulls (right).

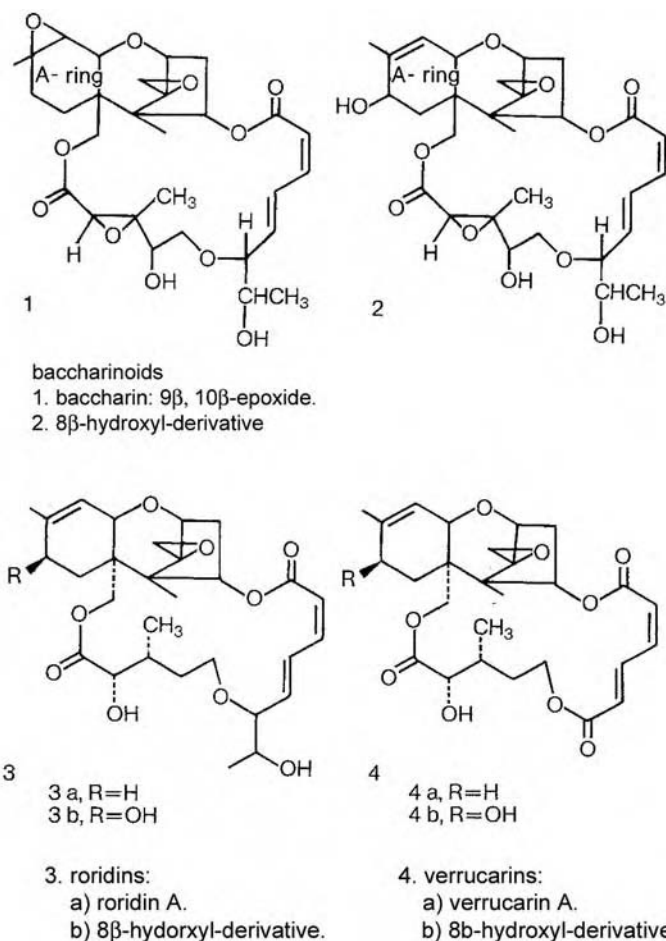
*Pseudomonas* sp. strain that is capable of using juglone as an exclusive source of carbon and energy.

### C. Trichothecenes of *Baccharis* Species

An unusual case of allelopathy is found in the Brazilian *Baccharis megapotamica* (Asteraceae) (14). Here the higher plant does not produce the active toxin independently, yet uptake and chemical modification occur. *Baccharis* sp. grow in its natural habitat only together with endemic grasses; other species are excluded. The plant delivers highly active antileukemic sesquiterpenes that belong to the group of trichothecenes. These compounds are macrocyclic antibiotics (roridins) that are normally produced by soil fungi, e.g., the saprophytic fungus *Myrothecium verrucaria*. The compounds isolated from *Baccharis* sp. are called *baccharinoids* (Fig. 8); they occur in significant amounts (0.02–0.03% of dry weight). Plants that are not growing in Brazilian soil do not contain baccharinoids. This difference is attributed to the absence of trichothecene-producing soil fungi.

The proof was provided by feeding experiments on *Baccharis* sp. seedlings with roridin A and the macrocyclic triester verrucaridin A, which





**Figure 8** Different trichothecenes with baccharinoids, roridins, and verrucarins.

both are rapidly taken up by the root system, transported to the shoot, and converted to the 8 $\beta$ -hydroxyl derivatives. A possible role of the macrocyclic trichothecenes may be regulation of reproduction and germination of Brazilian *Baccharis* species in their natural habitat (15).

However, investigations of Jarvis and associates (16–18) and his group indicate that trichothecenes in *Baccharis* sp. can also be produced by the plant itself. In any case, these compounds provide effective protection for the plant, not only against herbivores, but also against competing plants.

#### D. Communication Between Plants

The possibility of communication between plants was proposed about 20 years ago; few topics in plant ecology have inspired so much excitement and controversy. Karban and colleagues (19) use the term *plant communication* to indicate that “one plant changes its phenotypic level of resistance in response to environmental cues with no assumptions of benefit.” Now there is firm evidence that volatile compounds produced by plants, especially methyl jasmonate, are involved in interplant communication (20). Although the donor plant produces this substance as a response to herbivory, this process can be assessed as a type of allelopathy.

There are some recent examples that demonstrate this communication:

Damaged sagebrush (*Artemisia tridentata*) plants cause neighboring native tobacco (*Nicotiana attenuata*) plants to be more resistant to herbivores than tobacco plants located by undamaged sagebrush (19, 21).

Uninfested lima bean (*Phaseolus lunatus* cv. Sieva) leaves activate five separate defense genes when exposed to volatiles from conspecific leaves infested with spider mites (*Tetranychus urticae*), but not when exposed to volatiles from artificially wounded leaves (22). The expression pattern of these genes is similar to that produced by exposure to jasmonic acid.

At 10 different sites in Germany natural herbivory of single alder (*Alnus glutinosa*) trees by alder leaf beetle (*Agelastica alni*) increased with distance from one single defoliated tree (23). This result was confirmed by laboratory experiments. It reduces the possibility that associational resistance is responsible for the field result.

However, there is another important effect of the release of volatiles by plants after insect damage: these compounds can attract both parasitic and predatory insects that are natural enemies of the herbivores (24). This phenomenon is due to the presence of elicitors in the oral secretion of herbivores. One identified example is volicitin, *N*-17(S)-hydroxylinolenoyl-L-glutamine, present in the oral secretion of beet army worm (*Spodoptera exigua*), which was shown to elicit the release of plant volatiles that attract wasps that lay their eggs in the caterpillar (cf. Ref. 25). In addition to the plant volatile methyl jasmonate already mentioned, there is another component of plant volatiles induced by damage, *cis*-jasmone, or (*Z*)-jasmone (26). This compound is biosynthetically related to jasmonic acid and more volatile than methyl jasmonate.

Given the phenomenon of communication between plants there are still many questions remaining to be answered (21): does the change

in resistance caused by communication affect populations of herbivores? Does it provide any real advantage to the plants that have increased levels of resistance? Can communication affect the abundance and distribution of plants?

### III. ALLELOPATHY IN AGRICULTURE

There are numerous examples of allelopathic effects in agriculture and horticulture. On the positive side there are many crops that inhibit germination or growth of weeds during their lifetime, even as mulches. Some of them are listed in Table 1. On the other hand, there are numerous weeds that impair growth, especially of crops. Table 2 shows the complex interactions. It also contains examples of weeds that promote seed germination of crops.

**Table 1** Examples of Allelopathic Phenomena in Agriculture

Donor plant	Acceptor plant	Phenomenon	Participating allelochemicals <sup>a</sup>
Rye ( <i>Secale cereale</i> )	Weeds, e.g., <i>Chenopodium album</i> , <i>Avena fatua</i> , <i>Echinochloa crus-galli</i>	Reduction of weed biomass by mulches, growth suppression	Several, especially DIBOA, BO; phenolic acids (PLA, BHA)
Oat ( <i>Avena sativa</i> )	Corn poppy, mustard	Growth inhibition	Scopoletine
Wheat ( <i>Triticum aestivum</i> )	Camomile, mustard	Growth inhibition	Phenolic acids, simple acids, DIMBOA, DIBOA, MBOA
Sunflower ( <i>Helianthus annuus</i> )	Weeds with broad leaves; autotoxicity	Growth inhibition	Heliannuols
Alfalfa ( <i>Medicago sativa</i> )	Wild oats ( <i>Avena fatua</i> )	Displacement	

<sup>a</sup>DIBOA, 2,4-dihydroxy-1,4(2H)-benzoaxin-3-one; BOA, 2(3)-benzooxazolinone; MBOA, 6-methoxy-2-benzoxazolinone; DIMBOA, 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one; PLA,  $\beta$ -phenyl-lactic acid; BHA,  $\beta$ -hydroxybutyric acid.

**Table 2** Allelopathic Effects of Weeds

Donor plants	Acceptor plants	Phenomenon	Suspected allelochemicals
Bracken fern ( <i>Pteridium aquilinum</i> )	<i>Rubus parviflorus</i> , several grasses and shrubs	Inhibition of germination, reduced root growth	?
Quackgrass ( <i>Agropyron repens</i> )	Corn, alfalfa, common bean, soy bean	Chlorosis, growth inhibition, especially by plant residues; growth promotion	Tricin, p- hydroxybenzoic acid, vanillin
Field mint ( <i>Mentha arvensis</i> ) and corn poppy ( <i>Papaver rhoeas</i> )	Barley, rape	Promotion of germination, inhibition of germination	?
Corn cockle ( <i>Agrostemma githago</i> )	Rye, wheat	Promotion of germination and growth	Root exudations: agrostemine

### A. Autotoxicity (Asparagus and Apple)

Problems of growing the same crop in succeeding years due to poor establishment and stunting have led to investigations of possible causes, including allelopathy. Allelopathy that occurs among individuals of the same species is called *autotoxicity*. This phenomenon can often be observed within Chenopodiaceae (e.g., spinach), Cucurbitaceae (e.g., cucumber), Brassicaceae (e.g., cabbage), and Rosaceae (e.g., apple). Often an accumulation of pathogenic bacteria and fungi can be detected in combination with autotoxicity. In the following paragraphs asparagus and apple are discussed as representative examples.

#### 1. Asparagus (*Asparagus officinalis*)

Ten-year-old asparagus cultures already show a severe decline in quality and quantity. Therefore, this culture requires a resting period of another 10-yr. In the subtropical climate of Taiwan plantations must often be removed after 5 yrs. Fungicide treatment cannot completely replace this requirement (27).

Root exudates and plant residues are responsible for this phenomenon. Asparagus has an expanded root system with long storage roots that

are continuously produced and decay. The continued production of root biomass is a potential source of allelopathies. Plant residues inhibit root and sometimes shoot growth of asparagus seedlings (28). Several cinnamic acids have been identified as principal toxic components of asparagus, including methylenedioxycinnamic, ferulic, isoferulic, malic, citric, fumaric, and caffeic acid (29).

The damage is also due to indirect effects: when plant residues are ploughed in asparagus soils, infections with *Fusarium* sp. wilt are significantly increased (30). Another indirect effect, which can be attributed to the excretion of ferulic acid by asparagus, is the inhibition of hyphal growth of the arbuscular mycorrhizal fungus *Glomus fasciculatum*, which exists in symbiosis with asparagus. This inhibition results in reduced root colonization with the mycorrhizal fungus, reduced phosphorus absorption, and a general depression of root growth (31). Nonmycorrhizal plants did not show growth inhibition after application of ferulic acid.

## 2. Apple (*Malus domestica*)

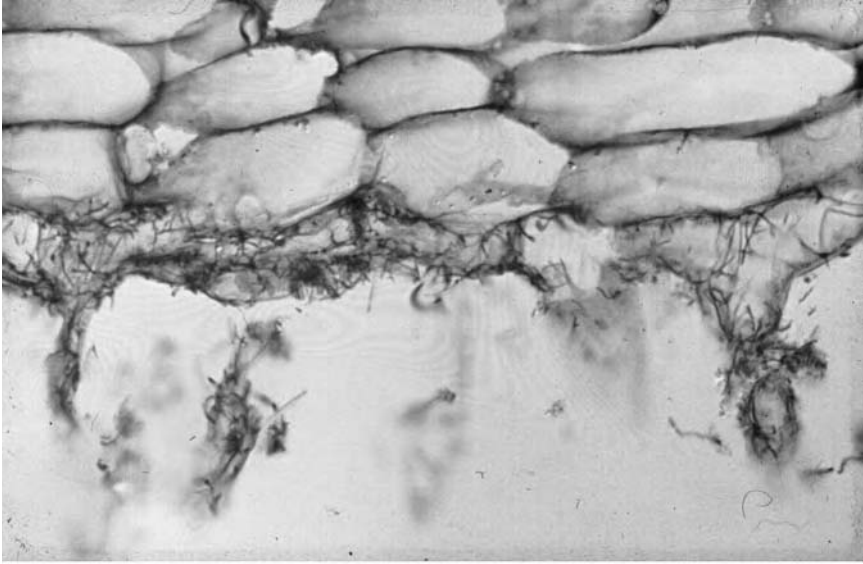
Poor growth of apple trees often occurs after replanting on a site that previously supported the same or closely related species. This phenomenon has been termed *replant disease* or *replant disorder* (32). Apple replant disease is widespread and has been documented in all of the major fruit-growing regions of the world (33). Symptoms include severe stunting, shortened internodes, rosetted leaves, small root systems, decayed or discolored roots, and reduced productivity (32). In the past apple replant disease was attributed to numerous abiotic factors, including unbalanced soil nutrition, heavy metal contamination, poor soil structure, cold or drought stress, low or high soil pH, and phytotoxins (cf. Ref. 32). Although all of these elements may be involved in tree growth problems, the fact that soil pasteurization dramatically improves plant growth (34) provides evidence that this disease is primarily a biological phenomenon rather than the result of abiotic factors. Figure 9 shows apple seedlings in field soil from an orchard replant site showing severe stunting (right) compared to gamma-irradiated soil from the same site (left). Several soil-borne organisms have been considered as potential causes of apple replant disease: plant-parasitic nematodes of the genus *Pratylenchus* (34), actinomycetes (35–37), fluorescent pseudomonads (38), and a number of fungi, including *Cylindrocarpum destructans*, *Phytophthora cactorum*, *Pytium* spp., and *Rhizoctonia solani* (32, 39). Winkler and Otto (40) excluded nematodes because the critical temperature to abolish replant disease is above the temperature tolerance of nematodes. The infection of apple roots with actinomycetes is shown in Fig. 10a–c.



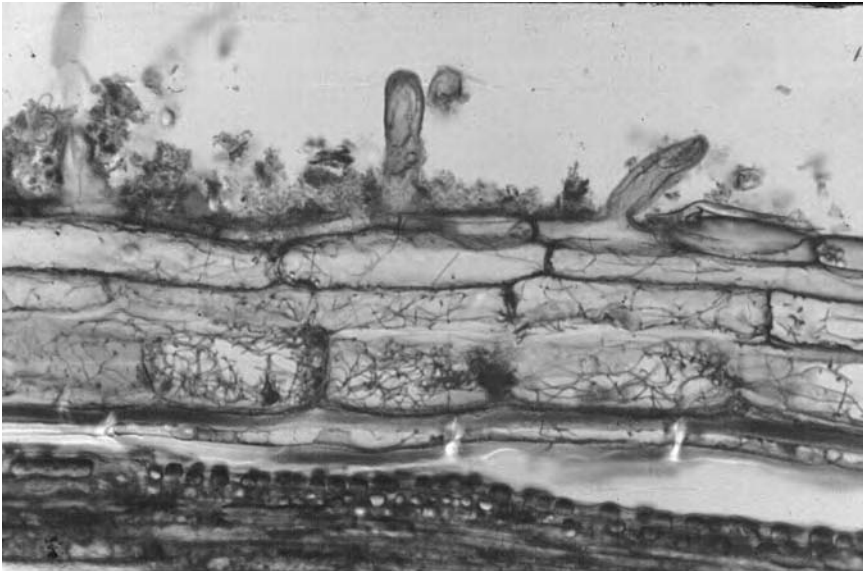
**Figure 9** Apple seedlings in apple replant disease soil (right) and in the same soil after sterilization (left). (Courtesy of L Wittenmayer, Martin-Luther-Universität Halle-Wittenberg, Germany.)

Results of Otto and coworkers (41) and Wittenmayer and Szabo (42) indicate that plant hormones are at least indirectly involved in the infection process.

In addition to the classical treatments, pasteurization and fumigation with biocides (32), there are different approaches to get rid of this



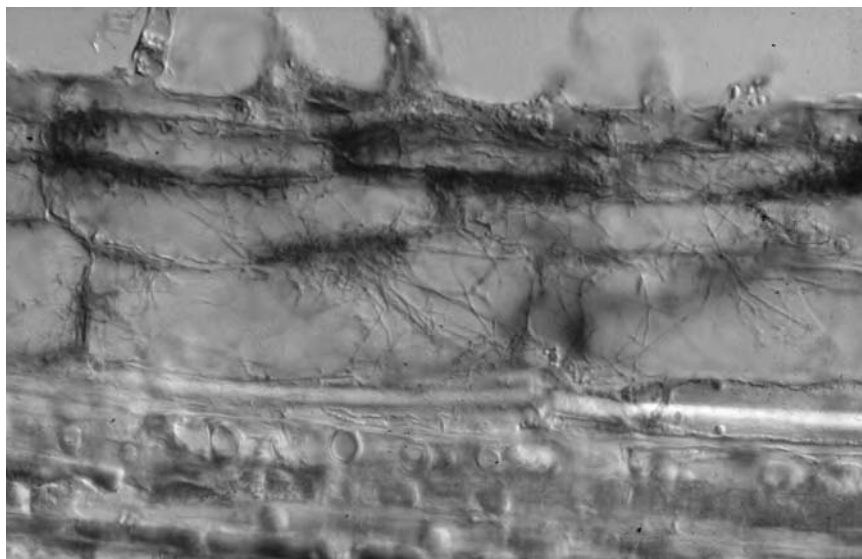
(a)



(b)

**Figure 10** a-c, Different infection stages of apple roots with actinomycetes. (Courtesy of G. Otto, Dresden, Germany.)





(c)

**Figure 10** Continued.

disease: in a growth chamber experiment root-lesion nematodes (*Pratylencheus penetrans* Filipjev) could be nearly eliminated after a marigold (*Tagetes patula* L. cv. Sparky) cover crop (34). Some *Bacillus subtilis* isolates showed inhibition or even significant antagonism against fungal strains that were isolated from apple replant disease soil (43). Inoculation of apple tree seedlings with several species of arbuscular mycorrhizal fungi suppressed phytotoxic micromycetes (44) and enhanced growth of young apple trees cultivated in apple replant disease soil (45).

There are several links of allelopathy to microorganisms:

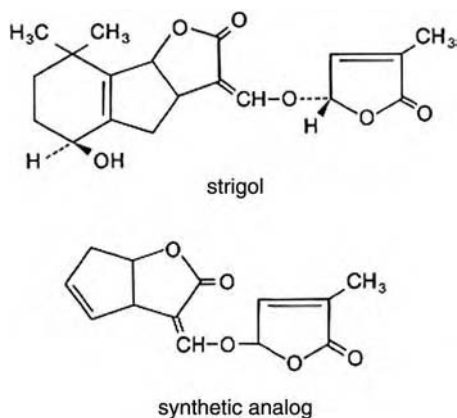
Weakening of plants of the same species by allelochemicals results in enhanced susceptibility to pathogenic microorganisms.

Allelochemicals are metabolites for microorganisms and promote their growth. Excretion products of these microorganisms weaken the higher plant.

The growth promotion of harmful organisms by allelochemicals leads to a displacement of useful microorganisms such as *Azospirillum* sp. or mycorrhizal fungi.

Soil microorganisms degrade allelochemicals and therefore reduce allelopathic effects.





**Figure 11** Strigol and a synthetic analog, both triggering the germination of *Striga* sp.

## B. Manipulation of Germination of Weed Seeds

The hemi- and root parasite of the genus *Striga* (Scrophulariaceae) provides an example of manipulation of weed seed germination. It invades members of the Gramineae such as maize, rice, sugar cane, and millet and leads to considerable damage of crops with dramatic effects on the human nutrition of wide parts of Africa (*Striga lutea*) and Asia (*Striga asiatica*). The danger is due to the high production rate of seeds and the long persistence of the seeds. Their germination is triggered by allelopathic substances excreted by the host plant.

In a rotational system the low specificity allows the cultivation of “wrong hosts” as catching plants (e.g., cotton). These plants trigger the germination of *Striga* sp. seeds, but they cannot be invaded (46, 47). The same effect can be evoked by ethylene or strigol (Fig. 11) that is excreted by roots of *Sorghum* and *Gossypium* sp. Synthetic analogs can be used as well. If the compounds are applied at the right time, *Striga* sp. seeds germinate but soon die because they cannot find a suitable host plant (48).

## C. Use of Allelopathic Interactions for Weed Control

Much work is still required before allelopathy can be used in agriculture as a routine approach. Nevertheless, several starting points for this purpose already exist:

Use of crop cultivars with allelopathic properties

Application of residues and straw from allelopathic crops as mulches

Use of allelopathic crop in rotational sequence where the allelopathic crop can function as a smother crop or where residues are left to interfere with the weed population of the next crop

Application of allelochemicals or modified allelochemicals as a kind of "natural herbicide"

Mixed cultivation of different crops

Breeding of highly allelopathic crops

The use of so-called natural pesticides, in this context natural, synthetic, or chemically modified allelochemicals, is different from the conventional use of herbicides. Strategies based on biological characteristics that result in maximal success together with minimal detrimental effect to the environment have to be elaborated.

#### IV. ECOLOGICAL EFFECTS OF ALLELOPATHIC PLANTS

Several allelochemicals provide a broad activity spectrum of biological activities. They are defense compounds against fungal and bacterial pathogens or insects (5). Allelochemicals may affect plant species through inhibition of their microbial symbionts such as mycorrhiza and nitrogen-fixing bacteria.

Seedlings of black spruce (*Picea mariana* Mill.) that grew close to *Kalmia angustifolia*, an ericaceous shrub with a high allelopathic potential, had a lower rate of mycorrhizal colonization and therefore significantly lower foliar concentrations of N and P than seedlings that grew far away from the shrub. Additionally the seedlings close to *K. angustifolia* were more frequently associated with *Phialocephala dimorphospora* Kendrick, a potential root pathogen of black spruce (49). This phenomenon leads to one of the major silvicultural problems in central Newfoundland. Here a failure of natural regeneration of harvested and burned areas and only poor growth of planted seedlings, particularly of black spruce, can be observed (50). Another example of the negative effect on mycorrhiza are laboratory and greenhouse experiments with seedlings of Scots pine *Pinus sylvestris*: aqueous extracts of crowberry *Empetrum hermaphroditum* reduced colonization with mycorrhizal fungi of the seedlings (51).

Compounds released from both living and herbicide-killed quackgrass (*Elytrigia repens*) inhibited the symbiosis between *Rhizobium* and legume species (52, 53). Laboratory studies indicated that compounds from quackgrass are particularly inhibitory to root hair formation; that inhibition subsequently prevents infection by *Rhizobium* sp. Compounds that inhibited

root hair formation did not inhibit the growth of several *Rhizobium* species. This means that the morphological changes induced by allelochemicals can reduce nodulation in legume species.

Apart from the direct toxic effect on other plants, some allelochemicals are thought to influence nutrient availability (cf. Ref. 9).

All these factors lead to the observation that the presence of allelopathic plants can affect population and community structures (cf. Ref. 9). Biodiversity can be reduced because genotypes sensitive to the allelochemicals are removed from the gene pool of the population by selection (54).

In summary, allelopathy may act directly on plants and other organisms and indirectly through change of soil properties, nutrient status, and altered population and/or activity of beneficial or harmful microorganisms, insects, nematodes, etc. (7). Therefore, allelopathic plants may have wide-ranging effects in ecosystems (9).

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### about the book . . .

The number of environmental compounds affecting plant metabolism and development is continuously increasing. This reference analyzes processes central to plant metabolism including uptake, distribution, and secretion of toxic material, and focuses on the recognition and prevention of damage associated with environmental pollutants.

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